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Introduction:

The twenty-first century warfighter will encounter the challenge of the potential use of toxic industrial chemicals (TICs) as chemical warfare agents. Large quantities of TICs are manufactured, stored, transported and used throughout the world. Many of these TICs are highly toxic and can rapidly affect exposed individuals causing severe injury and even deaths. Because TICs are stored and transported under relatively less secure conditions, these materials are easier for terrorists to inflict significant casualties against not only military but also civilian populations. In the event of a chemical attack, it will be vital to determine those individuals whose level of exposure would be anticipated to result in significant injury and possible death from those receiving only minimal or no exposure. The purpose of this project is to define the chemical signatures of the TICs acrylonitrile and acrolein in human blood, and potentially to devise a rapid, high throughput screening technology to enable examination of large groups of individuals following a known or suspected exposure. Studies have been directed toward defining the patterns of chemical reactivity of blood components with these TICs in order to use these adducts as biomarkers of chemical exposure. More recently experiments have focused on inventorying the tissue protein targets of acrylonitrile in order to gain insight into its mechanism of toxicity so that countermeasures (antidotes) may be developed.

Body:

Award Year 1:

The first three Technical Objectives stated on Page 7 of the Body of our Proposal were completed during the first year of the award period (October 1, 2010-September 30, 2011).

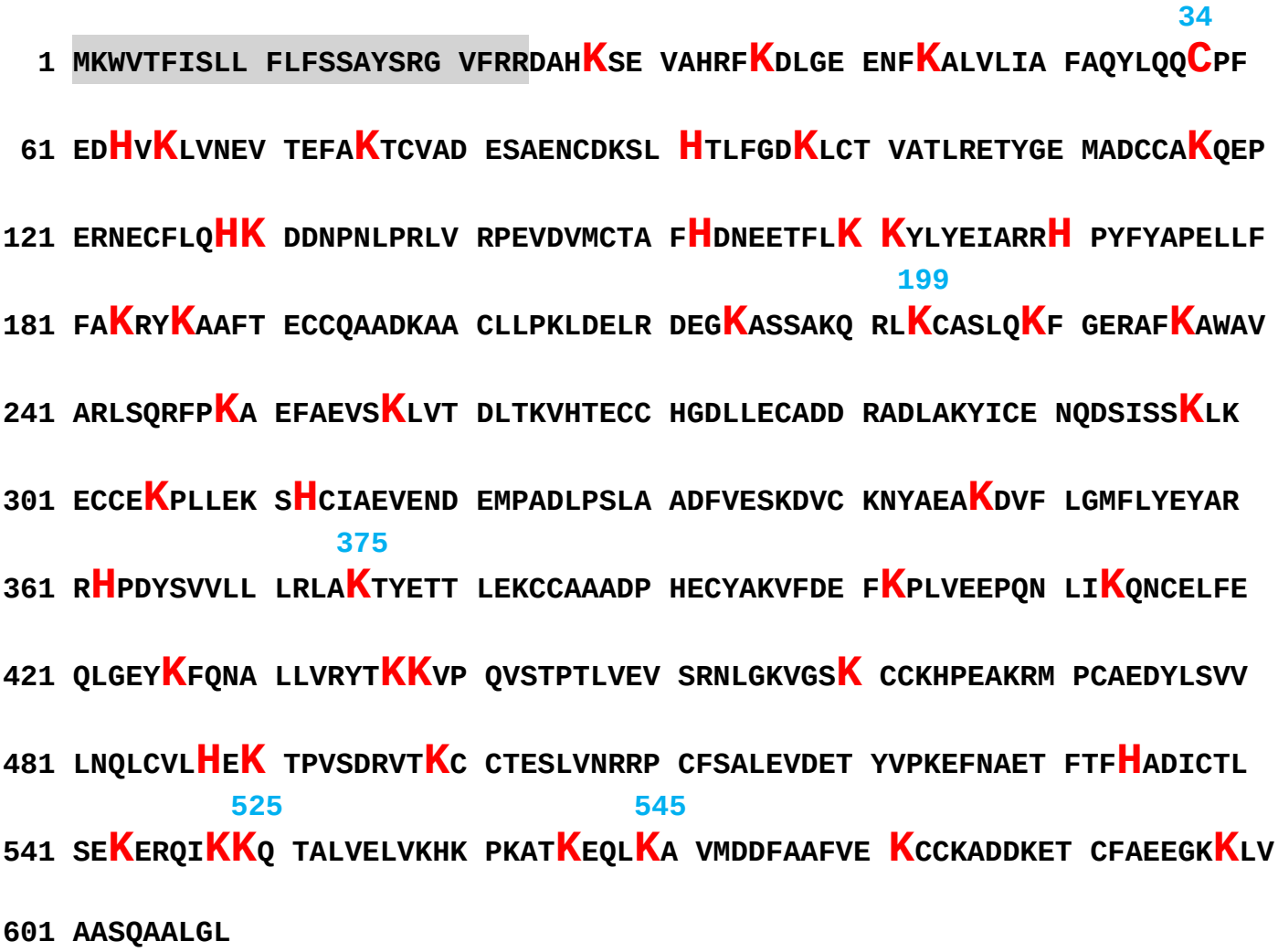
a) Characterize the biomarkers in human blood of exposure to the TICs acrylonitrile and acrolein. ***Question to be answered***: What are all the possible TIC-adduct sites in human blood, specifically on the abundant blood constituent's albumin, hemoglobin and glutathione.
b) Determine which of these TIC-blood-protein/peptide adducts (biomarkers) is most suitable for exposure monitoring for each TIC. ***Question to be answered***: Of the adducts detected in a), which is most suitable, from an analytical standpoint, for use as a biomarker of exposure.
c) Characterize the TIC blood concentration vs. adduct level response for these biomarkers in human blood. ***Question to be answered***: What is the relationship between the concentration of TIC in blood and the level of TIC biomarker measured.

Quarter 1-Award Year 1:

During the 1st Quarter of the 1st Award Year, we completed the analysis of all the possible TIC-adduct sites for acrylonitrile (AN) on human albumin. The methods used to accomplish this objective have previously been detailed on pages 2-8 of our quarterly report for the period October 1, 2010-December 31, 2010. The detailed results of those experiments were documented in three Tables on pages 9-11 of that report. A summary of the results of that work is shown below in Figure 1.

This report has color coding and thus may be best viewed or printed in color.

Figure 1: Human Serum Albumin Amino Acid Sequence and Sites of AN-adducts



HSA, as found in plasma, has 585 amino acids. The amino acid sites where cyanoethylation (AN-adduct) was detected are illustrated in the large red font.

It can be seen that AN reacted at multiple sites in the protein. 37 of the 59 lysine residues, 9 of 16 histidine residues but only 1 of the 35 cysteine residues formed adducts. Although cysteine is known to be highly reactive with AN, only one cysteine in HSA is free to react with AN as the other 34 are involved in disulfide bonds and thus protected from reaction.

This very high extent of reaction of HSA with AN is the result of the "harsh" conditions under which we conducted the experiments (100 mM AN at pH 7.4, 37°C for 24 h). We did this because the purpose of these experiments was to determine the "universe" of possible reaction sites in HSA.

To identify which of these 47 amino acid sites were the most reactive with AN, we measured those sites that reacted at very early time points in the incubation. As a result of those analyses, five amino acid sites were found to be the most reactive. The protein sequence numbers (ignoring the signal and propeptide portion) of five very reactive residues are shown above their one-letter amino acid code. They are Cysteine-34, Lysine-199, Lysine-351, Lysine-525 and Lysine-545. Their order of reactivity was estimated to be as follows:

Cys34 >>> Lys199>>Lys525>>Lys351>Lys545.

In the process of collecting and analyzing the HSA data, it became clear to us that because AN reacts not only at cysteine residues but also at lysine and histidine residues, that reaction at lysine would prevent trypsin cleavage at those lysine sites and thus complicate the calculation of the second-order rate constants at individual amino acid sites. This can be termed the "missed cleavage" problem.

In looking at the amino acid sequence of hemoglobin and the reactivity of AN, it occurred to us that collecting and analyzing the data for the reaction of AN with hemoglobin might provide a potential "work-around" to the "missed cleavage" problem (described below) and that hopefully what we learned in this analysis could then be applied to the more complex HSA data as well.

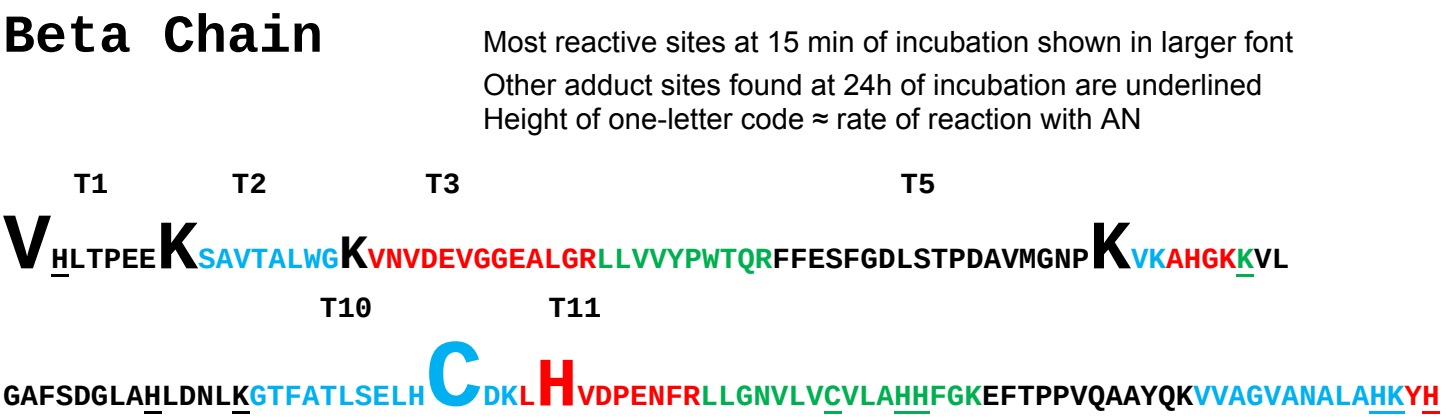
Quarter 2-Award Year 1:

Thus in the 2nd Quarter of the 1st Award Year, we presented our analysis of the reaction of AN with human hemoglobin (Hb), focusing on the five most reactive sites on the beta chain. This novel analysis allowed us to get estimates of the second-order rate constants for the reaction of AN at each of these five amino acid sites, including the lysines.

The methods used in these experiments were documented on pages 2-7 of our Quarterly Report for the period January 1, 2011-March 31, 2011. The "universe" of human Hb beta chain peptides identified subsequent to reaction with AN under the "harsh" conditions described above was presented in a Table on page 8 of that report.

Many of the peptides illustrated in that Table contained redundant information with respect to the sites of reaction of Hb with AN. The redundancy is removed in Figure 2 below, which shows the specific amino acid residues in Hb-beta chain where the cyanoethylation reaction was detected.

Figure 2: Human Hemoglobin Beta Chain Amino Acid Sequence, Tryptic Peptide Number and Sites of AN-adducts

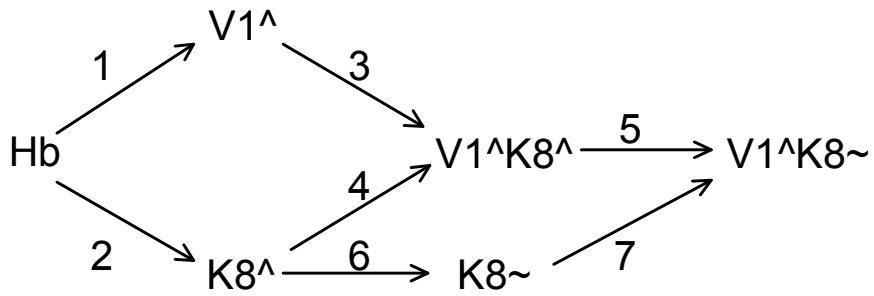


As mentioned earlier, and as illustrated above, AN reacts at histidine, lysine and N-terminal valine residues in addition to cysteine residues, which, on chemical grounds, would be expected to be the most reactive amino acid. AN reaction at lysine residues is especially problematic as the enzyme most often used for digestion of proteins for MS analysis is trypsin. Trypsin is chosen because it cleaves specifically at arginine and lysine residues creating a well-defined peptide digest. When AN reacts at lysine, trypsin cannot cleave at that site, so instead of getting a well-defined peptide digest, the digest will also include at least dipeptides due to "missed cleavages" at the reacted lysines and perhaps tri- and larger peptides depending on whether sequential lysines are modified. This creates a problem for measuring the second-order rate constants for the reaction of AN at not only these lysine sites but also at other reactive amino acid sites. This is best illustrated by considering the reaction of AN with V1 and K8 in the hemoglobin beta chain.

When control hemoglobin is digested with trypsin, peptide T1 (Figure 2) from the beta chain is produced due to trypsin cleavage at K8. If AN only reacted at V1, then monitoring the amount of peptide T1V1^ (peptide T1 with a single AN-adduct at V1) as a function of time would allow calculation of the second-order rate constant for the reaction of AN at βV1 in human Hb. However, when hemoglobin is reacted with AN, we find that it reacts not only with βV1 but also with βK8. Peptide T1V1^ will not form if AN has reacted at K8, which it does to a significant extent. Thus the amount of T1V1^ formed will depend on the extent of reaction with AN at both βV1 and βK8. Similar considerations apply at the other sites on the beta chain that react with AN.

The following scheme illustrates the complexity of the kinetics.

Figure 3:



The second-order rate constants that we wanted to measure are represented by arrow 1 (rate of reaction of AN with V1 to form V1^) and arrow 2 (rate of reaction of AN with K8 to form K8^). As can be seen from the above scheme, these initial products react further (arrows 3 and 4) to produce the double adducted product V1^K8^. In addition, there is a parallel reaction where K^ can react further with AN to form K8~, the dicyanoethylated product (arrow 6). The terminal tricyanoethylated product V1^K8~ can form via further hemoglobin reaction with AN represented by arrows 5 and 7. The presence of all of these products was detected by mass spectrometry when hemoglobin was reacted with AN over 24h.

The ideal approach for monitoring the kinetics of the appearance of each product would be to have internal standards synthesized for each product so that individual response factors could be applied to the measured adduct peptide peak areas as the reaction with AN progresses. However, since our purpose was only to rank the relative reactivity of the AN-reactive sites in human blood to identify the one most reactive site for use as a biomarker of exposure, we used a "label-free" approach to estimate the second-order rate constant of appearance of AN-adducts. In addition, we were able to quantitatively measure the disappearance kinetics, as described on pages 12-17 of our Quarterly Report for the period January 1, 2011-March 31, 2011 of each unreacted tryptic peptide as the response factor is not required.

The methods and detailed results of these experiments were previously described on pages 12-20 of our Quarterly Report for the period January 1, 2011-March 31, 2011. Some of those results were refined via subsequent experiments. Table 1 shows the estimated second-order rate constants for the reaction of AN with Hbβ V1, K8 and K17 based on the disappearance of unreacted peptides T1, T2 and T3.

Table 1								
Second-Order Rate Constants ($M^{-1}min^{-1}$) for the Reaction of AN with Hbβ V1, K8 and K17 Calculated from the Second-Order Rate Constants for the Disappearance of Hbβ Peptides T1, T2 and T3								
	T3/T4 Disappearance			**T2/T4 Disappearance**			**T1/T4 Disappearance**	
AN+Hb	**f [k(K17)]**			**f [k(K8)+k(K17)]**			**f [k(V1)+k(K8)]**	
	k(K17)			**k(K8) calc.**			**k(V1) calc.**	
				(k8+k17) - k17			[k(V1)+k(K8)] - k(K8)	
Mean	**1.2E-03**			**6.1E-03**			**2.1E-02**	

The method used to estimate the rate constants listed in Table 1 were documented on page 9 of our 1st Annual Report dated October, 2011.

We also estimated the rate of reaction at these same three sites by measuring the rate of appearance of the adducted peptides. For this analysis, we only used the initial time points of the reaction before subsequent reactions on the same amino acid site occurred. These rates are dependent on unknown response factors for the adducted peptides illustrated in Figure 3. However, making the assumption that the response factors for the adducted products were not significantly different from the un-adducted peptides allowed us to estimate the second-order rate constants of for the rate of reaction to form K17^, K8^ and V1^ as 1.9 E-03, 6.3 E-03 and 2.1 E-02, respectively. These values are in good agreement with those calculated from the disappearance kinetics and did not change the relative order of reactivity that was our desired goal.

Because of the good agreement between the rates calculated from appearance of product and disappearance of unreacted peptide described above, we estimated the second-order rate constants for the reaction of AN with K59, C93 and H97 based on product appearance. The results are shown in Table 2 below.

Table 2								
Second-Order Rate Constants ($M^{-1}min^{-1}$) for the Reaction of AN with Hbβ K59, C93 and H97 Calculated from the Rate of Appearance of Hbβ Peptides T5K59^, T10C93^ and T11H97^								
	T5K59^ Appearance			**T10C93^ Appearance**			**T11H97^ Appearance**	
AN+Hb	**f [k(K59)]**			**f [k(C93)]**			**f [k(H97)]**	
Mean	**5.3E-03**			**4.0E-02**			**5.1E-03**	

Using the approach referenced above for estimating the rates of reaction from the disappearance curves of unreacted peptides T5, T10 and T11, we calculated the second-order rate constants for

the reaction at K59, C93 and H97 as 3.3 E-03, 3.7 E-02 and 4.6 E-03, respectively. Again, there is reasonable agreement between the two approaches.

Table 3 is a summary of the results for the Hb beta chain.

C93 > V1 > K8 > K59 ≈ H97 > K17

Table 3					
Second-Order Rate Constants for the Reaction of AN with Human Hemoglobin β-Chain ($M^{-1}min^{-1}$)					
C93	**V1**	**K8**	**K59**	**H97**	**K17**
4.0E-02	2.1E-02	6.1E-03	5.3E-03	5.1E-03	1.2E-03

We subsequently used the same methodology described above to complete the analysis of the second-order rate constants for the reaction of AN with sites on the Hb alpha chain.

Quarter 3-Award Year 1:

The methods used in those experiments were documented on page 2 and pages 4-13of our Quarterly Report for the period April 1, 2011-June 30, 2011. The "universe" of human Hb alpha chain peptides identified subsequent to reaction with AN was presented in a Table on page 3 of that report.

Many of the peptides illustrated in that Table contained redundant information with respect to the sites of reaction of Hb with AN. The redundancy is removed in Figure 4 below, which shows the specific amino acid residues in Hb-alpha chain where the cyanoethylation reaction was detected.

Figure 4: Human Hemoglobin Alpha Chain Amino Acid Sequence, Tryptic Peptide Number and Sites of AN-adducts

Alpha Chain

Most reactive sites at 15 min of incubation shown in large font
Other adduct sites found at 24h of incubation are underlined
Height of one-letter code ≈ rate of reaction with AN

T1 T2 T3 T4

VLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHGKKVADALTNAVAHVDD
MPNALSALSDLHAHKLRVDPVNFKLLSHCLLVTLAAHLPAEFTPAVHASLDKFLASVSTVLTSKYR

The second order rate constants for the disappearance of unreacted peptides T1 to T4 via reaction with AN are shown below in Table 4.

Table 4											
Second-Order Rate Constants for the Disappearance of Tryptic Peptides αT1,αT2, αT3 and αT4 ($M^{-1}min^{-1}$)											
	αT1/βT4 Disappearance			**αT2/βT4 Disappearance**			**αT3/βT4 Disappearance**			**αT4/βT4 Disappearance**	
AN+Hb	**[k(V1)+k(K7)]**			**[k(K7)+k(K11)]**			**[k(K7)+k(K16)]**			**[kK16)+kH20)]**	
Mean	**2.8E-02**			**8.6E-03**			**6.6E-03**			**3.5E-03**	

In addition, we were able to estimate the second-order rate constants for the reaction of AN with alpha Valine-1 (V1^) and alpha Histidine-20 (H20^) from their appearance kinetics listed below in Table 5.

Table 5					
Second-Order Rate Constants for the Reaction of AN with Hbα V1 and H20 ($M^{-1}min^{-1}$)					
	V1^ Appearance			**H20^ Appearance**	
AN+Hb	**[k(V1)]**			**[k(H20)]**	
Mean	**2.3E-02**			**2.5E-03**	

These values were then used sequentially to estimate the second-order rate constants for the reaction with AN with the three lysine residues (K7, K11 and K16) lying between V1 and H20. The detailed methodology was presented on page 12 of our 1st Annual Report dated October, 2011.

The rates estimated for the alpha chain reactive sites in human hemoglobin are shown in Table 6.

Table 6				
Second-Order Rate Constants for the Reaction of AN with Human Hemoglobin α-Chain (M-1min-1)				
V1	**K7**	**K11**	**H20**	**K16**
2.3E-02	5.0E-03	4.6E-03	2.5E-03	1.0E-03

The order of reactivity for the 5 most reactive sites on the alpha chain of human hemoglobin is as follows:

$$V1 > K7 \approx K11 > H20 > K16$$

Combining the order of reactivity of the beta chain with the order of reactivity of the alpha chain yields the complete order of reactivity of the most reactive sites in human hemoglobin with AN.

βC93 > αV1 ≈ βV1 > αK8 ≈ βK59 ≈ βH97 ≈ αK7 ≈ αK11 > αH20 > βK17 ≈ αK16

The slowest second-order rate constant measured was 1.0 E-3 $M^{-1}min^{-1}$ for αK16. The order of reactivity at the other sites relative to this slowest site is as follows:

Table 7		
Site of AN-Adduct	$M^{-1}min^{-1}$	Relative Rate
βC93	4.0E-02	40.0
αV1	2.3E-02	23.0
βV1	2.1E-02	21.0
βK8	6.1E-03	6.1
βK59	5.3E-03	5.3
βH97	5.1E-03	5.1
αK7	5.0E-03	5.0
αK11	4.6E-03	4.6
αH20	2.5E-03	2.5
βK17	1.2E-03	1.2
αK16	1.0E-03	1.0

As can be seen, the most reactive site for AN-adduction in human hemoglobin was βC93. Its rate of adduction was approximately twice that of the N-terminal valines, which have previously been used as a biomarker of AN exposure.

Thus the amino acid site on human hemoglobin that is most reactive with AN is Cysteine-93 on the beta chain with a second-order rate constant of 4.0 E-02 $M^{-1}min^{-1}$. Although this reaction rate is reasonably high, it is over three hundred times slower than the second-order rate constant for AN reaction with Cysteine-34 of human albumin, which we have measure as 13.5 $M^{-1}min^{-1}$ from previous studies.

That being the case, we did not see any value in conducting extensive additional experiments to estimate the far slower reaction rates for the four less reactive lysine sites in HSA as mentioned on page 6 of this report.

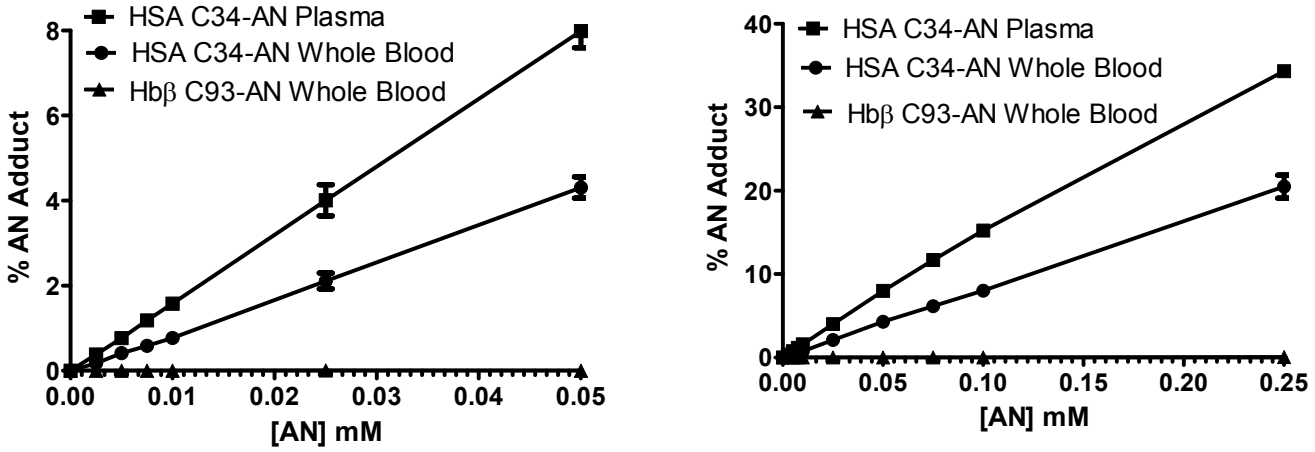
Thus the biomarker that we employed to develop a rapid assay for human AN exposure is the C34-AN adduct of HSA.

Quarter 4-Award Year 1:

In the 4th Quarter of the 1st Award Year, we focused exclusively on the reactivity of HSA C-34. We first conducted studies on the AN-concentration dependence of the first-order reaction rate constant. This was necessary to insure that the first-order rate constants observed was directly proportional to the AN concentration. This verified that we were working under pseudo first-order conditions and that the second-order reaction rate constants that we calculated by dividing the observed first-order rate constants by the AN concentration (100mM) was valid. The results of those experiments were documented on page 14 of our 1st Annual Report dated October, 2011.

In addition, the second major investigation conducted during this period was determining the relationship between the level of HSA-C34-AN adduct product as a function of AN concentration. The purpose of these experiments was two-fold. First to estimate the lowest level of detection and second to establish the relationship between AN exposure and HSA C34-AN adduct level for biomarker purposes. The detailed results of those experiments were documented on pages 14-16 of our 1st Annual Report dated October, 2011. Some of those results are shown below in Figure 5.

Figure 5: Concentration Dependence of AN-adduct Formation-Linear-Scale (expanded ranges)



The data indicate that the most reactive cysteine in hemoglobin, namely Hbβ C93 (Table 7) is an incredibly poor biomarker of AN exposure compared to HSA C34; the % AN-adduct barely rising above the abscissa even at the highest AN concentration.

The reason why HSA C34-AN adduct levels are higher in plasma incubations vs. whole blood incubations is due to competition reactions for AN with Hb reactive sites and GSH in whole blood, that are absent in plasma incubations. Previous studies indicate that whole blood GSH is quite reactive with AN but its second-order rate constant is only one third that of HSA C34.

To be a useful biomarker for monitoring acute AN-exposures in humans, it must be sensitive enough to detect low-level non-toxic exposures up to and including toxic exposures. It is clear from Figure 5 that HSA C34-AN adduct levels can be detected and quantified in whole blood-AN incubations with AN concentrations as low as 0.0025mM. Previous studies in our laboratory have shown that a peak AN blood level of 1-2mM following a subcutaneous injection in rats is consistent with severe AN toxicity, including possible lethality. Thus it is anticipated that we could detect human exposures at approximately 1000 times less than a lethal blood level in rats.

Award Year 2:

In the second year of the Award we accomplished the fourth and fifth Technical Objectives of the proposal:

d) Conduct studies aimed at minimizing the biomarker analysis time so that the method will be suitable for triage purposes. **Question to be answered:** What analytical steps used for TIC biomarker level measurement can be accelerated or possibly eliminated in order to minimize analysis time.
e) Characterize the acrylonitrile blood concentration vs. adduct level response for its' biomarker in rat blood. **Question to be answered:** What is the reactivity of rat blood vs. human blood for the acrylonitrile biomarkers of interest.

In Award Year One we definitively established that HSA-C34-AN levels can serve as a sensitive biomarker of acrylonitrile (AN) exposure in humans that can be used for triage purposes following a chemical attack or industrial or transportation accident involving acrylonitrile.

Quarter 1-Award Year 2:

During the first quarter of this second year, we conducted experiments toward optimizing the analysis time so that multiple samples could be analyzed in the minimum amount of time.

There were two steps in the analysis of HSA-C34-AN adduct in a human blood sample that were amenable to optimization. One was the trypsin digestion time and the other was the Liquid Chromatography (LC) time. Experiments directed toward optimizing the LC analysis time were documented in detail in the Quarterly Report for the period October 1, 2011-December 31, 2011.

The report indicated that a complete RPLC run of a peptide digest would take about 45 minutes. Thus, including the other steps required to prepare the blood sample for RPLC analysis, it would take well over one hour to analyze one sample. This was clearly too long to be of use in analyzing multiple samples for triage purposes.

The obvious solution was to see if adduct quantification could be achieved by infusing the entire plasma protein peptide digest directly into the mass spectrometer bypassing the RPLC step completely. We documented that this was indeed possible by using the Z = 2 ion clusters of peptides T5-IAA and T5-AN (pages 4-8, Quarterly Report for period October 1, 2011-December 31, 2011).

To increase the accuracy of the quantification, we applied a small correction factor to account for the overlap of the M+5 peak of T5-ANwith the M+1 peak of T5-IAA. This small correction amounted to 6.1% and the corrected height of the M+1 peak of T5-IAA could be calculated with the following equation.

Height of M+1 of T5-IAA = Height of m/z 1246.15 – Height of M+1 of T5-AN (m/z 1244.15) * 0.061

Having established the appropriate ions to monitor in order to quantify the level of AN-adduction to HSA by infusion of a plasma protein tryptic digest directly into the mass spectrometer, we conducted experiments designed to optimize the infusion solvent conditions. Those experiments were described in detail on pages 8-10 of the Quarterly Report for the period October 1, 2011-December 31, 2011. The conclusions from those experiments were that the optimum infusion rate for a plasma digest was 25 ul/min, the optimal solvent strength of the infusion was 50% acetonitrile. Electrospray, capillary and tube voltages were also optimized.

From the results described above we established that we could eliminate the time-consuming RPLC step by directly infusing the plasma protein digest directly into the mass spectrometer and assess AN-adduct levels quantitatively.

The next most time consuming step in the analysis is the time required to digest the plasma protein sample with trypsin. Typically digestion of plasma or tissue proteins with trypsin is conducted over several hours or in some cases overnight. This time scale would not be suitable for rapid triage of exposed individuals.

We had previously done some preliminary experiments using various techniques that had been published in the literature claiming to speed up the digestion. These included adding acetonitrile or urea to the digestion mixture. Although neither addition was found useful in our hands, those experiments did indicate that certain segments of HSA could be digested very easily whereas other regions require more time to be digested completely and that, fortunately, Cys-34 happens to be located in a region that is more easily digested. As has been pointed out in earlier reports, for our purposes, we require complete digestion of HSA in order to utilize the T5-adduct at Cys34 as a quantitative biomarker of the level of exposure to a toxic industrial chemical. This is a far more stringent requirement than the extent of digestion needed for the purpose of protein identification.

---------T4----------|----------**T5**----------|----------T6----------|

DLGEENFKALVLIAFAQYLQQ**C**PFEDHVKLVNEVTEFAK

Quantification of AN-adduction on HSA **C**34 requires that peptide T5 be completely liberated from the rest of the protein for analysis. Thus trypsin must hydrolyze the peptide bond at both the T4-T5 and T5-T6 junctions, i.e. there must be no missed cleavages.

Experiments were conducted monitoring the disappearance of peptides T4- T5and T5-T6, indicting the hydrolysis of these peptide bonds by trypsin, as a function of digestion pH, time and temperature as described on pages 10-13 of the Quarterly Report for the period October 1, 2011-

December 31, 2011. The conclusion of these experiments was that digestion was complete within 15 minutes at pH 8.5 at 50°C.

Having now optimized the two rate-limiting steps in the analysis of a blood sample for the quantification of AN-adduction, we applied these techniques to the analysis of a series of fresh human blood, which had been treated with AN in the concentration range 0.0025 – 2.0 mM.

We analyzed these samples by both the "Gold-Standard", but time consuming RPLC method, and by the newly developed "Fast-Analysis" approach. The detailed results of those analyses were presented on pages 13 and 14 of the Quarterly Report for the period October 1, 2011-December 31, 2011. A summary table comparing the results of the Fast analysis vs. the "Gold-Standard" RPLC analysis of T5-AN adduct levels in AN-treated fresh human whole blood is shown below.

Table 8:

Sample	AN (mM)	T5-AN (%)	
		RPLC	Fast
B3-00	0	0.00	0.11
B3-01	0.0025	0.18	0.06
B3-02	0.005	0.47	0.08
B3-03	0.0075	0.57	0.19
B3-04	0.01	0.72	0.34
B3-05	0.025	1.84	1.63
B3-06	0.05	3.89	3.35
B3-07	0.075	5.14	5.94
B3-08	0.1	7.21	7.50
B3-09	0.25	17.83	19.03
B3-10	0.5	32.86	35.34
B3-11	0.75	46.38	48.51
B3-12	1	55.47	58.31
B3-13	2	69.47	73.25

The data indicate that the Fast analysis method gives good agreement with the RPLC method and thus would be suitable for triage purposes. It should be noted that the method is capable of detecting blood levels of human AN exposures approximately 1000 times less that the lethal blood level in rats.

Reproducibility of the Fast analysis method was checked on two blood samples one at a low and one at a high adduct level and was found to produce excellent reproducibility.

As a result of the experiments outlined above, we were able to establish a reasonable Time Line for the analysis of multiple samples for the triage of exposed individuals. One such possible Time Line is tabulated below.

Table 9:

Fast Analysis Time Line:

Order of Events	Procedure	Time min
1	Sample arrives in Lab and is logged into data system	
2	Transfer from 0.2-2.0ml blood to microfuge tube and centrifuge 13K-g	1
3	Add 20ul of plasma supernatant to 1ml acetonitrile in	
4	Centrifuge 13K-g to pellet plasma proteins	1
5	Suck off acetonitrile supernatant	
6	Dissolve plasma protein pellet in 250ul 50mM Ambic, pH 8.5	1
7	Add 20ul above to 20ul of 4mM DTT in 50mM Ambic, pH 8.5	
8	Incubate above at 70°C to reduce protein disulfide bridges	10
9	Add 5ul of 50mM IAA at room temperature to alkylate protein cysteines	5
10	Add 150ul, 50mM Ambic and 4ul Trypsin (0.5ug/ul) to digest proteins at 50°C	15
11	Add 50ul, 0.5% Formic acid to stop digestion	
12	Transfer 200ul of sample to LC sample vial containing 200ul ACN	
13	Start sample infusion into Orbitrap MS and begin data acquisition	5/sample
14	Conversion of raw MS data into % albumin adduction	

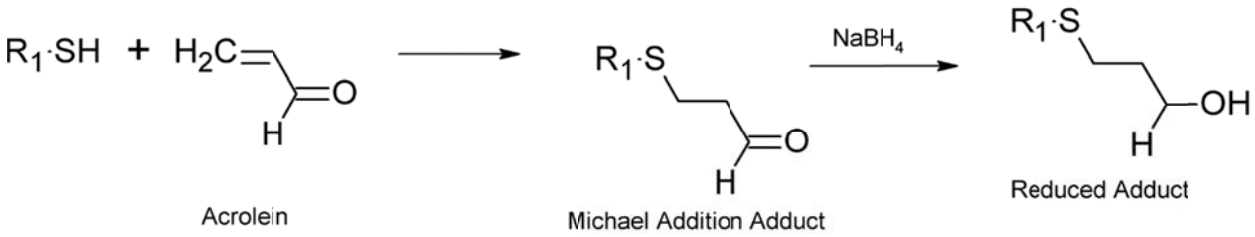
Ambic: Ammonium Bicarbonate
DTT: Dithiothreitol
IAA: Iodoacetamide
ACN: Acetonitrile

Some of the times in minutes listed in the table are fixed in that centrifugation (steps 2 and 4) require 1 min, disulfide bridge reduction (step 8) requires 10min, alkylation of the reduced cysteines (step 9) takes 5min and trypsin digestion (step 10) takes 15 min. These times are fixed but multiple samples can be processed in parallel so the times are the same for processing 1 or multiple samples. The sample infusion and data acquisition time (step 13) and the conversion of the raw MS data into a % albumin adduction can only be done serially and thus are on a per sample basis. The steps where no time is indicated can typically be accomplished in less than one minute each and some in parallel. We estimate that it would be possible using the above Time Line to conservatively process 10 blood samples in less than two hours with one mass spectrometer. Throughput would obviously be proportional to the number of mass spectrometers available. Recall that the method we have developed does not require the high resolution and thus relatively expensive instrument that we used for these studies and thus equipping a laboratory with multiple lower resolution mass spectrometers capable of this analysis is not unreasonable.

Quarter 2-Award Year 2:

Our main objective for the Award Period January 1, 2012-March 31, 2012 was to begin work on the second toxic industrial chemical that we have proposed to study, namely acrolein. Acrolein adduction analysis was anticipated to be much more complex than acrylonitrile because of its higher chemical reactivity, its bifunctional reactive groups (aldehyde and vinyl) and the instability of its initial reaction products with amino acids in proteins. The

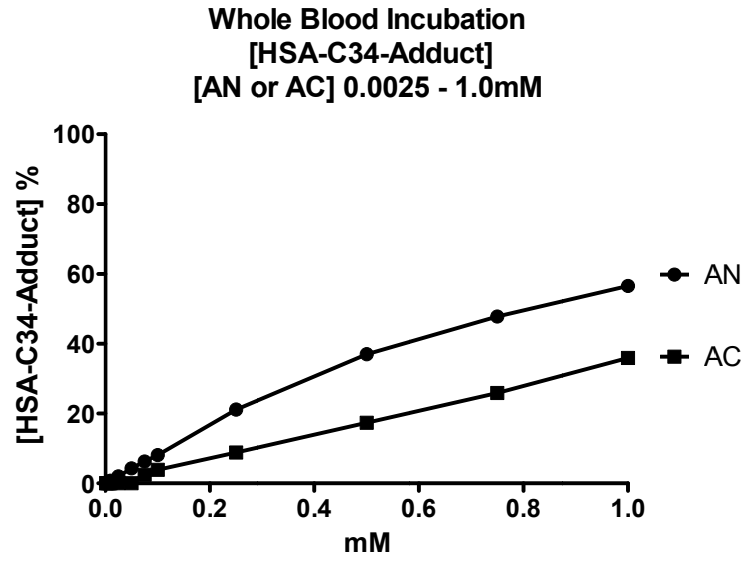
chemistry underlying this complexity was illustrated on pages 2-3 of the Quarterly Report for the period January 1, 2012-March 31, 2012.
From our previous studies of the reaction of acrylonitrile (AN) with human blood, we anticipated that AC, like AN, would be highly likely to form initial Michael adducts with human serum albumin Cys-34 (HSA-C34), glutathione (GSH) and human hemoglobin β Cys-93 (Hbβ-C93). To minimize, or hopefully eliminate, the potential subsequent reaction of the initial Michael addition adducts with amino groups, we used sodium borohydride to reduce the reactive aldehyde group to the more stable alcohol. This would preclude secondary complicating reactions.



During this quarter we developed methods for measuring acrolein (AC) adducts of HSA-C34, GSH and Hbβ-C93 in whole blood incubations with acrolein as we had previously done for acrylonitrile (AN). Details were provided on pages 4-8 of the Quarterly Report for the period January 1, 2012-March 31, 2012.

We then used these methods to compare the concentration dependence of HSA-C34-Adduct formation in whole blood for acrolein as compared to acrylonitrile. The results of one such experiment is shown below.

Figure 6:



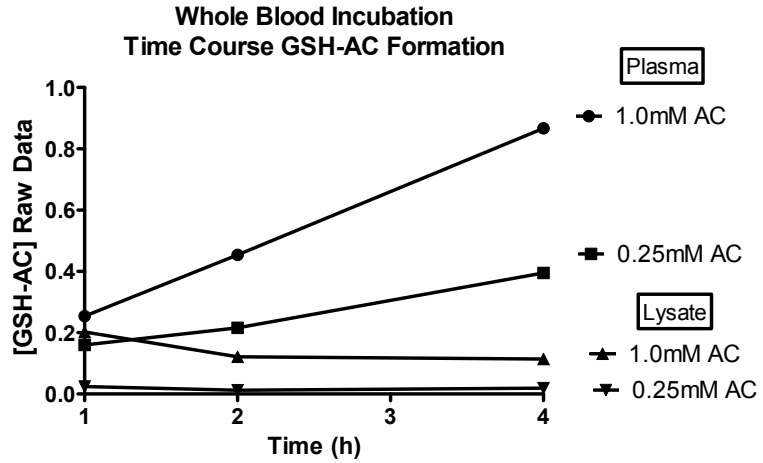
The data indicated that HSA-C34-AC adduct formation in reactions with whole blood was considerably less than that found in similar incubations with AN. This suggests that there may

be some component in whole blood that is reacting with AC even faster than it reacts with HSA-C34 thus consuming AC. This other component apparently does not react or reacts much more slowly with AN. GSH and Hbβ-C93 are present in whole blood but unlikely to be responsible for this difference. Further investigations into this phenomenon were postponed as they were considered to be tangential to the current Statement of Work.

In order to monitor AC alkylation of GSH in whole blood, we synthesized the GSH-AC adduct and a stable isotope labeled analog $^{13}C_2$,^{15}N-GSH-AC, which would serve as an internal standard for quantification. Details of those experiments were presented on pages 10-14 of the Quarterly Report for the period January 1, 2012-March 31, 2012.

We subsequently used this methodology to measure the depletion of whole human blood GSH and the formation of the GSH-AC adduct as a biomarker of AC exposure. The results of one such experiment is illustrated below.

Figure 7:



Despite the fact that virtually all blood GSH is inside the red cell (≈ 2mM), whereas the plasma GSH concentration is in the low μM range, when we assayed for GSH-AC adduct levels we found higher levels of adduct in plasma than in the red cell lysate. One possible explanation might be that the AC reacted with GSH in the red cell but the adduct was then pumped out into the plasma. If this were the case, one still might expect higher adduct levels in the red cell unless the pump was incredibly fast and efficient.

The second startling finding was that the plasma GSH-AC adduct levels continued to increase over the 4h incubation period. This might not be surprising were it not for the fact that in the exact same incubation, the HSC-C34-AC adduct level was not changing over the same time period This is indeed a puzzling finding and further experiments will be needed to clarify this phenomenon. Again these experiments were postponed as they are also tangential to the Statement of Work.

As shown in previous reports, Hbβ-C93 reacts with AN and thus is expected to form adducts with AC as well. For AN, the second-order rate constant for its reaction with Hbβ-C93 is some

300-times slower than its reaction with HSA-C34. Thus we might expect a similar difference with AC. Experiments, as described on pages 14-17 of the Quarterly Report for the period January 1, 2012-March 31, 2012 bore this out.

In summary, as expected, monitoring the reaction of AC with the whole blood components, albumin, GSH and hemoglobin was more complex that with AN. It may not be possible to measure the second-order rate constants for the reaction of AC with these three blood components as we did for AN because of the speed of the reaction. However, this does preclude using the HSA-C34-AC adduct as a biomarker for assessing AC exposure. The cause and resolution of some of the unexpected observations that we made in working with AC, particularly with GSH, are of more academic than practical interest. As illustrated in Figure 6, the HSA-C34-AC adduct is clearly a measure of AC exposure.

Quarter 3-Award Year 2:

One of our major technical objectives was to characterize the acrylonitrile blood concentration vs. adduct level response for AN biomarkers in rat blood. The rationale was that since we have previously established the relationship between the acrylonitrile dose, the blood acrylonitrile-adduct level and the resulting toxicity in rats in vivo, we proposed to conduct in vitro studies with rat blood as done for human blood. Comparing the in vitro human vs. rat blood acrylonitrile concentration-biomarker level data will allow us to estimate the human toxicity expected for a given level of human blood biomarker. This knowledge will be incredibly useful for triage purposes. During this quarter, our animal care facility had informed us that they would be sacrificing sentinel animals over the next several months and thus could provide us with fresh rat blood needed for these experiments.

GSH & GSH-AN Adduct Measurements in Rat Whole Blood:
Since rat GSH is identical to human GSH, the methods that we employed to measure the reactivity of AN with GSH in whole rat blood were identical to those that we used for whole human blood. Those methods were described in detail on pages 4-7 of the previously submitted Final Report for W81XWH-08-1-0047, dated August 2010 and will not be repeated here.

Rat Albumin-AN Adduct Measurement in Rat Plasma:
In previous studies we have shown that the most reactive site in human albumin for reaction with AN is Cysteine-34 (C34). Rat albumin (RSA) is 80% homologous with human albumin and also contains C34 as its only free cysteine. The other 34 cysteine residues are involved in 17 disulfide bridges as in human albumin. Thus we anticipated that C34 would be the most reactive site in rat albumin as well.

There is however an important amino acid sequence difference near C34 in rat albumin that affects the tryptic peptide in which C34 is located.

```
          T1--|-T2----|T3|---T4----|------T5---------|----T6----|
Rat       EAHKSEIAHRFKDLGEQHFKGLVLIAFSQYLQKCPYEEHIK
          T1--|-T2----|T3|---T4----|-----------T5-------------|
Human     DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVK
```

Unlike human albumin, where **C34** is located in a large tryptic peptide T5, in rat albumin, **C34** is located in the much smaller peptide T6. Unfortunately, this decreases the sensitivity in the detection of AN-adducts at this amino acid site.

The methods used to monitor the reaction of AN with rat albumin are identical those used for human albumin. These methods were described in detail on pages 7-11 of the previously submitted Final Report for W81XWH-08-1-0047, dated August 2010 and will not be repeated here.

Typical results of using those methods for monitoring the reaction of AN with RSA-C34 were detailed on pages 3-6 of the Quarterly Report for the period April 1, 2012-June 30, 2012.

Rat Hemoglobin-AN Adduct Measurement in Rat Red Blood Cell Lysate:
In previous studies we have shown that the most reactive site in human hemoglobin for reaction with AN is Hbβ-Cysteine-93 (βC93). See page 17 of the Annual Report dated October 2011.

Unlike human Hb, the rat Hb tetramer contains extra cysteines located at βC125 and αC13, of which the former is exposed, whereas the latter is completely buried within the polypeptide chain. βC125 is known to have a low pKa, a high accessibility and, consequently, a high reactivity. In fact, previous studies in our laboratory administering 14C-labeled AN to rats indicated that βC125 was the only site of AN-adduction to Hb in vivo. Thus we anticipated that βC125 would be highly reactive in our in vitro incubations with AN.

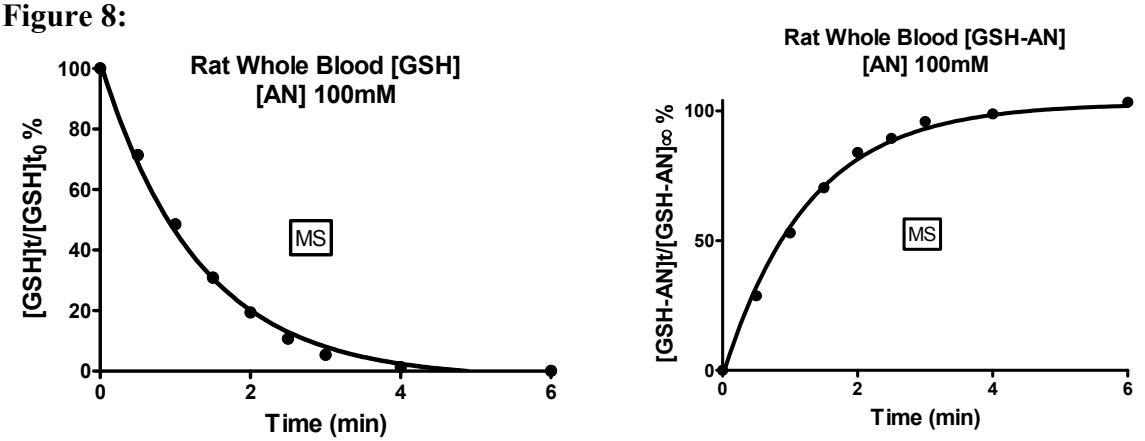
The methods used to assess AN-adducts on rat hemoglobin were identical to those used for human hemoglobin and were described in detail on pages 11-14 in the previously submitted Final Report for W81XWH-08-1-0047, dated August 2010 and will not be repeated here.

Typical results of using those methods for monitoring the reaction of AN with RSA-C34 were detailed on pages 6-9of the Quarterly Report for the period April 1, 2012-June 30, 2012.

Second Order Rate Constants for the In Vitro Reaction of the TIC Acrylonitrile with the Most Reactive Sites in Rat Blood:
Having now devised methods for the measurement of AN-adducts of rat blood GSH, albumin-C34 and Hbβ-C93 and C125, and having verified that these sites are the most reactive nucleophiles in blood for the TIC acrylonitrile, we needed to put the relative reactivity of AN with these blood constituents on a quantitative basis. To do this we measured the second order rate constants for the *in vitro* reaction of AN with rat hemoglobin, albumin and GSH.

Rate Constants for the Reaction of AN with GSH in Rat Whole Blood:
Typical plots of the normalized concentration ratios as a function of time for the disappearance of GSH and the appearance of the GSH-AN adduct determined by Mass Spectrometry (MS) are illustrated below.

Figure 8:



In a similar way, the rate constant for the disappearance GSH was measured spectrophometrically using the colorimetric reagent DTNB, which forms a yellow colored product upon reaction with GSH. A typical plot of the disappearance of GSH as a function of time of the reaction of 100mM AN with whole blood using the spectrophotometric method was illustrated on page 11 of the Quarterly Report for the period April 1, 2012-June 30, 2012.

These experiments were repeated on a total of four separate days several months apart. The second order rate constants calculated from this data are presented below.

Table 10:

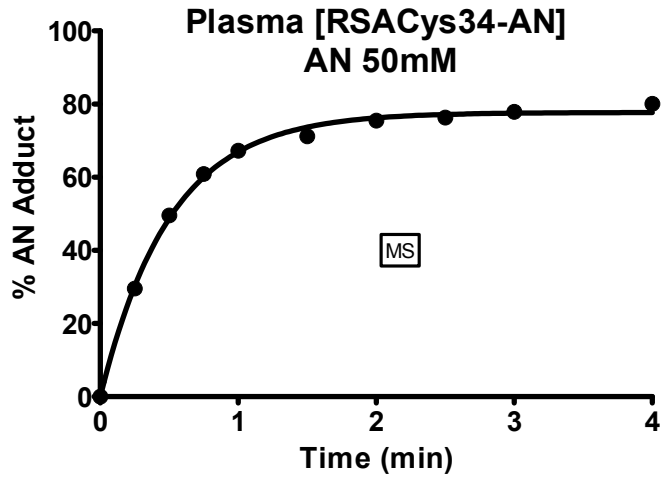
Second Order Rate Constants ($M^{-1}min^{-1}$ ± S.D.)		
↓[GSH]	↓[GSH]	↑[GSH-AN]
Spectrophotometric	Mass Spectrometry	Mass Spectrometry
6.7 ± 0.2	6.6 ± 0.4	6.7 ± 0.4

Note the excellent agreement between the two techniques for measuring the disappearance of GSH and the agreement of both with the rate of appearance of the GSH-AN adduct.

Rate Constant for the Reaction of AN with RSA-C34 in Rat Plasma:
Ion Chromatograms (monitoring the Z = 3 and Z =2 ions of peptides T6 and T6* (Figure 2 page 4, Quarterly Report for the period April 1, 2012-June 30, 2012) were constructed from plasma tryptic digest samples obtained at various times during the reaction of rat plasma with AN at 37°C, pH 7.4-7.5. A typical set of ion chromatograms were illustrated on page 12 in our Quarterly Report for the period April 1, 2012-June 30, 2012.

These area changes as a function of time were used to calculate the second order rate constant for the appearance of the RSA-C34-AN adduct. Typical plot of the area changes as a function of time for the appearance of the RSA-C34-AN adduct determined by mass spectrometry are illustrated below.

Figure 9

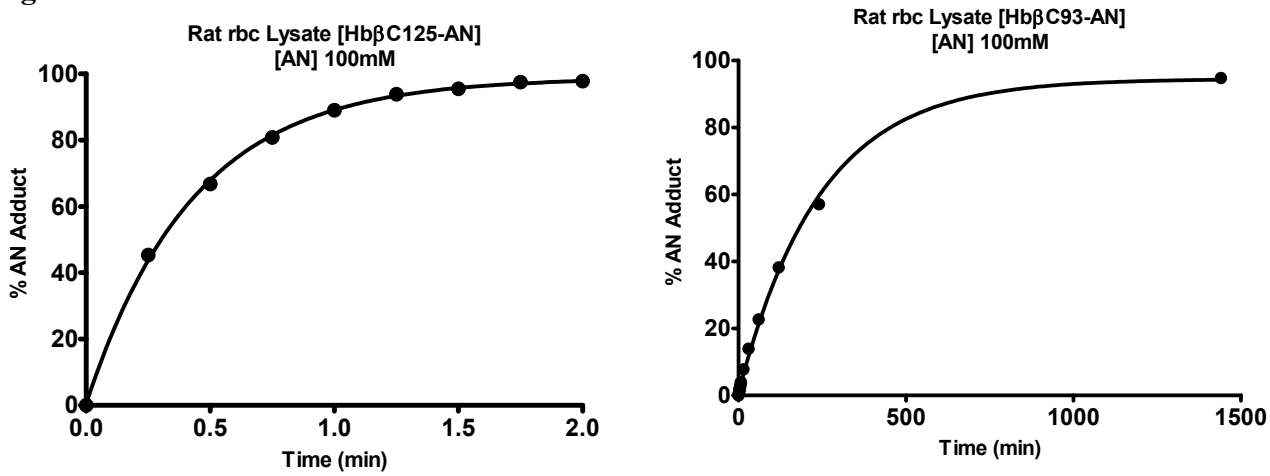


The second order rate constant for the appearance of RSA-C34-AN adduct in this individual experiment was 39.5 $M^{-1}min^{-1}$. The average rate constant for this reaction for all of the experiments we have conducted will be summarized after the following description of the reaction of AN with human hemoglobin in RBC lysates

Rate Constants for the Reaction of AN with HbβC93 and βC125 in Rat RBC Lysates: The rate of formation of the AN-adducts of HbβC93 and βC125 was measured in rat RBC lysates as illustrated on pages 13-15, Quarterly Report for the period April 1, 2012-June 30, 2012.

The area changes as a function of time for both adducts were used to calculate the second order rate constants for the appearance of the HbβC125-AN and HbβC93-AN. Typical plots of the area changes as a function of time for the appearance of these adducts determined by mass spectrometry are illustrated below.

Figure 10:



The second order rate constants for the appearance of HbβC125-AN and HbβC94-AN adducts in these individual experiments were 0.041 and 23.2 M-1min-1, respectively.

Summary of the Rat Rate Constants and Comparison to the Human Rate Constants for the Reaction of AN with the Reactive Sites in Blood.

The table below is a summary of the rate constants measured for the reactive sites in rat blood measured during this quarter with the equivalent rate constants previously measured in human blood.

Table 11:

Second Order Rate Constant						
k $M^{-1}min^{-1}$ (Mean±S.D.)						
	↓ [GSH]	**↓ [GSH]**	**↑ [GS-AN]**	**↑Alb-C34-AN**	**↑Hbβ-C93-AN**	**↑Hbβ-C125-AN**
	Spec.	MS	MS	MS	MS	MS
Human	4.4±0.6	4.3±0.5	4.1±0.5	11.8±1.5	0.040±0.012	NA
Rat	6.7±0.2	6.6±0.4	6.7±0.4	36.3±2.6	0.041±0.001	23.9±0.3

In general, the rate constants measured in rats were faster than those measured in human blood, except for HbβC93, which had the same reactivity in both species. Although this site is the least reactive of those listed in the table, recall that it actually is the most reactive site in human hemoglobin (page13, Annual Report, October 2011). As stated earlier, however, rat hemoglobin has two additional cysteine residues not found in humans. One of them, βC125 was found to be some 600 times more reactive than βC93 with AN.

The most reactive site observed was found to be rat albumin C34. We had previously observed that human albumin C34 was the most reactive site in human blood but did not expect the same site in rat blood to be approximately 3 times more reactive. It is known that the protein structure surrounding C34 in human albumin creates a highly reactive environment. The higher reactivity of this site in rat albumin would suggest that its environment is even more favorable for reaction with AN and perhaps other electrophiles.

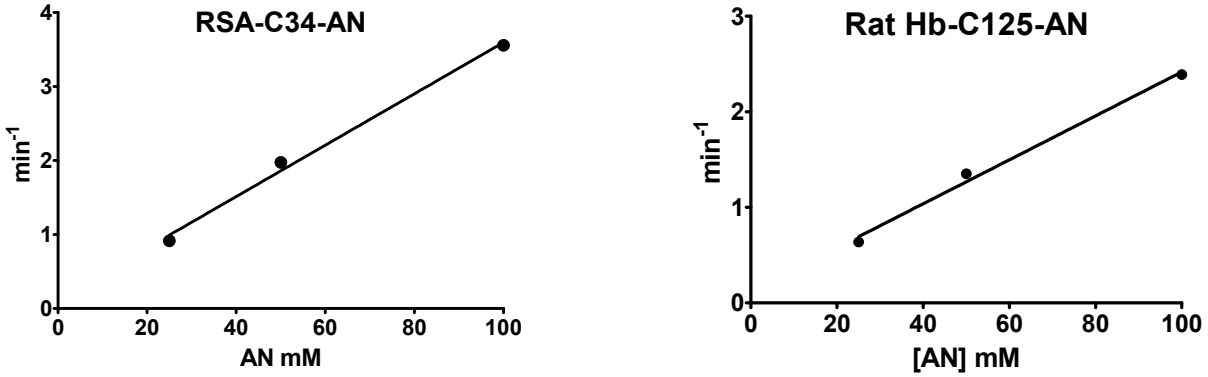
The higher reactivity of GSH in rat blood than in human blood was also unexpected. Since GSH is a small molecule with no secondary or tertiary structure, one would anticipate that its chemical reactivity would be identical in both species. At least two possibilities might be responsible for this observation. First, since essentially all of blood GSH is inside the red cell, it is possible that the rat RBC creates a more favorable environment for reaction. Alternatively, reaction of GSH with AN is known to be catalyzed by glutathione-S-transferase (GST) and differences in GST content in rat vs. human RBCs might exist.

Some effort may be invested in rationalizing these unexpected findings but the main goal of this quarter was to quantify the relative reactivity of rat vs. human blood with AN and that goal has been accomplished. This relative blood reactivity data combined with known levels of toxicity of AN in rats with rat blood adduct levels will enable prediction of potential toxicity to be expected in humans based on human blood adduct levels.

Quarter 4-Award Year 2:

We first conducted studies on the AN-concentration dependence of the first-order reaction rate constants measured in the previous quarter. This was necessary to insure that the first-order rate constants observed are directly proportional to the AN concentration. This would verify that we were working under pseudo first-order conditions and that the second-order reaction rate constants that we calculated by dividing the observed first-order rate constants by the AN concentration (100mM) were valid.

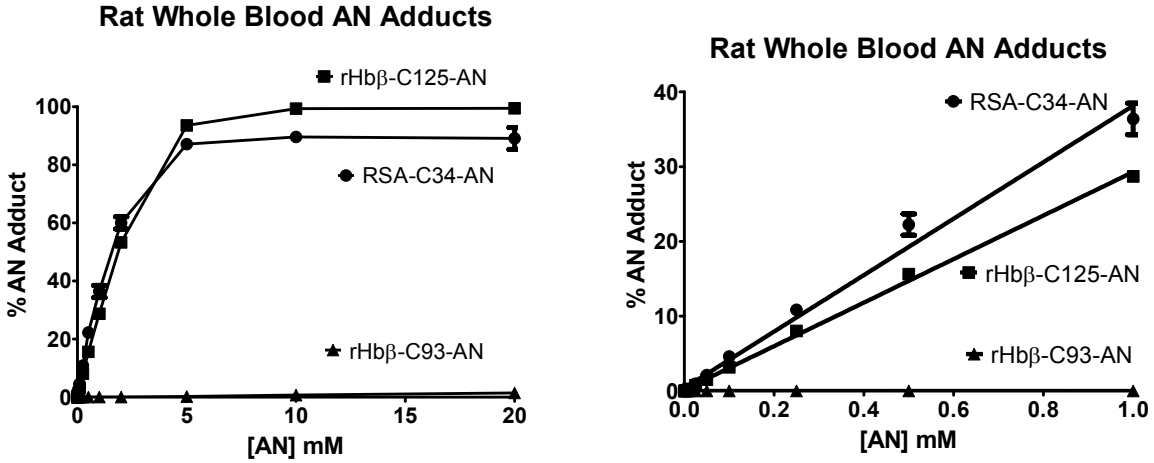
Figure 11:



As shown in Figure 11, we observed, as expected, a linear relationship between the first-order reaction rate constants for the reaction of AN with RSA-C34 and rat Hb-C125 over a 4-fold concentration range. This verified the second-order reaction rate constants summarized in Table 11.

The second major investigation conducted during this quarter was determining the relationship between the levels of RSA-C34-AN. rat Hb-C125-AN and rat Hb-C93-AN adducts as a function of AN concentration. The purpose of these experiments was two-fold. First to estimate the lowest level of detection and second to establish the relationship between AN exposure and these adduct levels for biomarker purposes. The results of those experiments are illustrated below.

Figure 12:

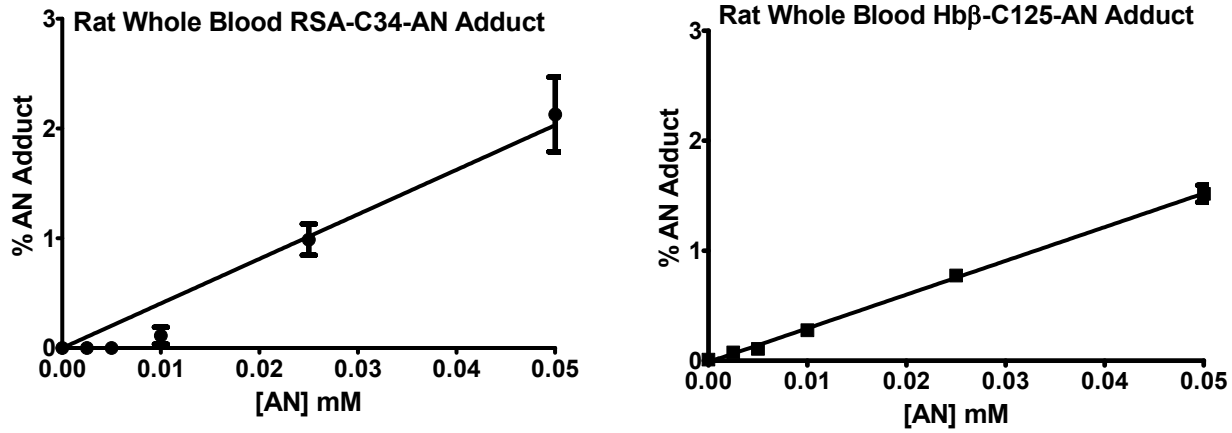


As can be seen in Figure 12 (left), the % AN adduct increases as a function of the concentration of AN in the incubation for both of the most reactive sites in rat blood, namely RSA-C34 and rHbβ-C125. Both sites have reacted completely within 1 hour at all concentrations above 5mM AN. The % adduct at Hbβ-C125 reaches 100% whereas the RSA-C34 only reaches 85-90% because, as with human albumin, a certain percentage of C34 in blood is unavailable for reaction with AN as the C34 thiol is not free but exists as a mixed-disulfide with GSH.

In contrast, the % adduct at rHbβ-C93-AN at 1 hour at 20mM AN is only about 1.5%. This is the result of its second-order rate constant for reaction with AN being almost 1000-fold slower than the other two reactive sites (Table 11).

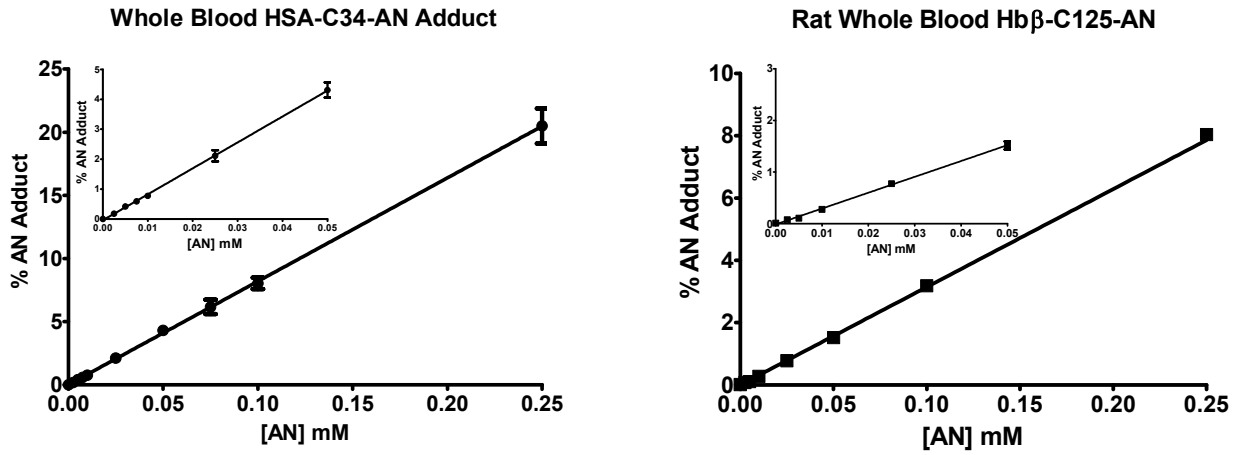
Linear regression analysis in the range of 0 - 1.0mM AN for the two most reactive sites indicated no significant deviation from linearity (Figure 12, right). However this was misleading when looking at the data more carefully in the lowest AN concentration range.

Figure 13:



As can be seen in Figure 13 (left), although linear regression analysis of RSA-C34-AN adduct formation did not significantly deviate from linearity over the range 0 - 1.0mM AN, the intercept of this line was not 0% AN adduct at 0mM AN and no adduct was detected at the two lowest concentrations of AN used (0.0025 and 0.005mM AN). This was not the case for the Hbβ-C125-AN adduct (Figure 13, right). The reason for the non-ideal behavior observed for RSA-C34-AN was alluded to earlier. Recall that unlike the HSA-C34-AN adduct, which is found on the large 21 amino acid T5 peptide, RSA-C34-AN is found on the smaller 8 amino acid peptide T6. Peptide T6 elutes much earlier in the RPLC run, which for technical reasons results in decreased sensitivity compared to later eluting peptides. Peptide T14, containing Hbβ-C125-AN, elutes much later in the chromatogram and does not suffer from this loss in sensitivity. Thus the rHbβ-C125-AN adduct is a better biomarker for AN exposure in rats than is RSA-C34-AN, especially in the low exposure range, whereas in humans the HSA-C34-AN adduct is the better biomarker for human exposure. Figure 14 below compares these two biomarkers of AN exposure.

Figure 14:



The data in Figure 14 complete Technical Objective d) originally stated on Page 7 of the Body of our Proposal. Although, in the future, we anticipate having a much more detailed description of the use of these data to predict the human toxicity to be expected at a given level of HSA-C34-AN adduct measured in a human with our technology, we can already state the following. Administering a subcutaneous dose of 20mg/kg of AN to a rat is just below the threshold dose of acute toxicity. This dose will produce a peak blood level of AN of approximately 0.15mM AN. We can detect HSA-C34-Adducts in whole blood exposed to 0.0025mM AN, some 60-fold lower than this threshold blood level for acute toxicity in rats. Assuming for the moment equal sensitivity of rats and humans for AN toxicity, it is clear our method has adequate sensitivity to assess human exposure to this toxic chemical from well below toxic levels to up to and through 100% lethal levels (≈ 1mM AN).

Nine Month No-Cost Extension (NCE) October 1, 2012-June 30, 2013

Quarter1-NCE:

Our objective for this quarter was to assess the rHbβC125-AN adduct levels in rats that had been treated with AN in vivo. This objective was accomplished as is illustrated in the summary below.

Earlier in this project we measured the rate constants for the covalent reaction of the toxic industrial chemical acrylonitrile (AN) with the most reactive sites in human blood. Those studies indicated that the most reactive site was HSA-C34.

However, in order to estimate the expected human toxicity that might be associated with a measured level of HSA-C34-AN adduct in a human blood sample, a Human Adduct Level vs. Human Toxicity calibration curve is required. Producing such a calibration curve by exposing human subjects to toxic levels of AN is not ethically possible.

However, such toxicity studies have been done in rats and, if the relationship between the relative reactivity of AN with rat blood components vs. human blood components was known, then we could use a Rat AN-Adduct Level vs. Rat Toxicity calibration curve to estimate the expected human toxicity for a given level of AN-adduct in humans.

We determined and tabulated this relationship between the relative reactivity of AN with rat blood components vs. human blood components on page 24, Table 11.

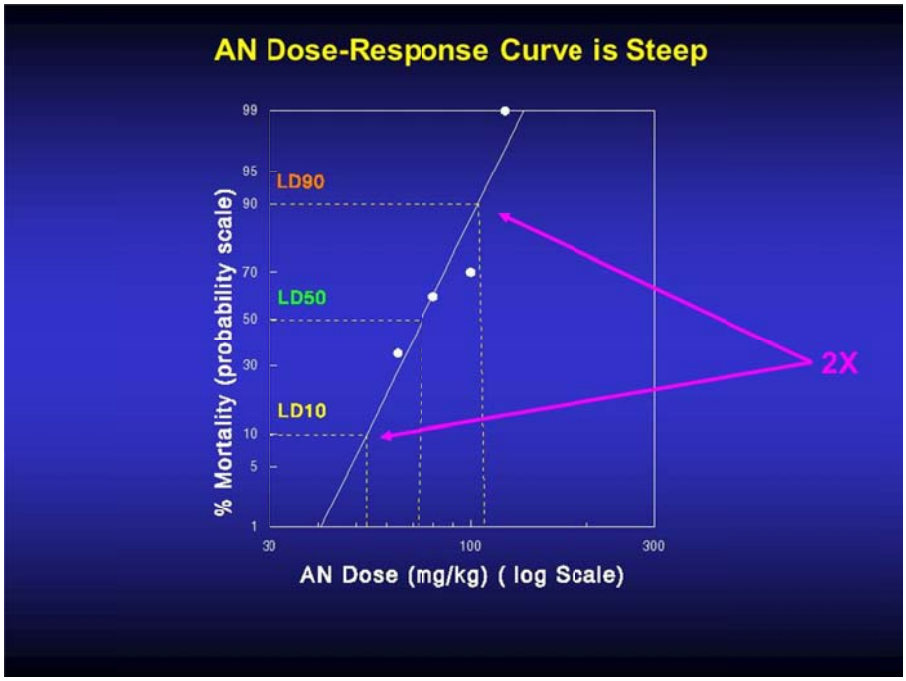
These results indicated that Cysteine-34 in rat albumin was the most reactive site in rat blood as it was in human blood. In fact the RSA-C34-AN adduct formed three times faster than the HSA-C34-AN adduct. Thus the RSA-C34-AN adduct in rat blood could be used as a biomarker of AN exposure in rats just as the HSA-C34-AN adduct could be used in humans.

In addition, unlike human hemoglobin, where Hbβ-C93 is very unreactive toward AN, as it is in rats as well, rats have an additional cysteine residue at rHbβ-C125 that is nearly as reactive with AN as is RSA-C34. Thus **either** the RSA-C34-AN adduct **or** the rHbβ-C125-AN adduct could be used to assess exposure to AN in rats.

<u>AN Toxicity in Rats:</u>
We have been investigating the mechanism of the acute toxicity of AN for over 25 years. During that period we have conducted several studies that are critically related to the current project and specifically to the results to be presented for this quarter.

We had previously characterized the Dose-Response Curve for the acute toxicity of subcutaneous administration of AN in rats. This Dose-Response curve for acute lethality is illustrated below.

Figure 15:

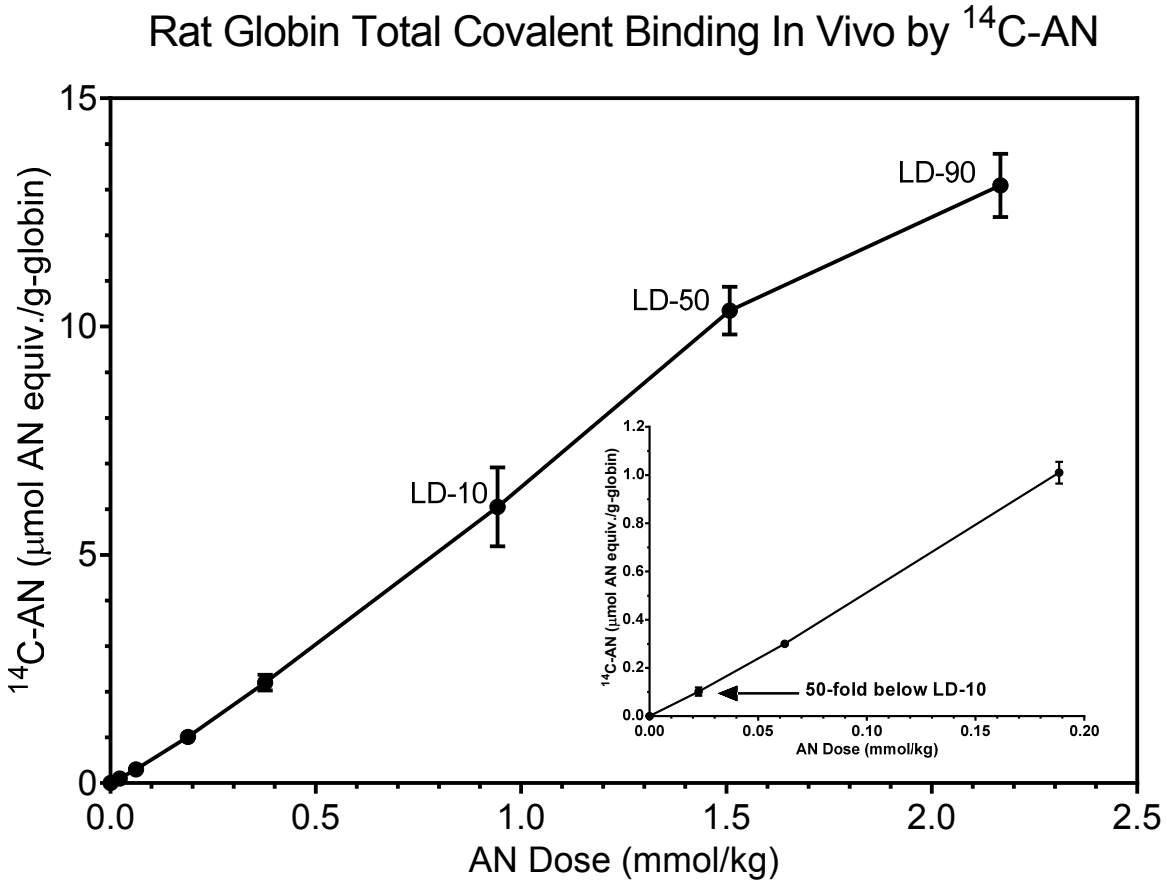


From the above graph the LD-10, LD-50 and LD-90 were determined to be 50mg/kg (0.94mmol/kg), 80mg/kg (1.51mmol/kg) and 115mg/kg (2.17mmol/kg), respectively. In addition, it was noted that the slope of the Dose-Response curve was incredibly steep, in that the LD-90 was only two times (2X) higher than the LD10. If AN were acting via a receptor binding mechanism, the Law of Mass Action would predict a 81 times increase in dose would be required to go from an LD10 to and LD-90 Obviously, this is not the case.

<u>Previous Measurements of AN-adduct Levels in Rats Treated with AN Doses Illustrated in Figure 15 and Lower Doses:</u>

In experiments we conducted over 15 years ago, using ^{14}C-labeled AN, we measured the level of ^{14}C-AN-globin adducts at each of the three acutely toxic doses listed above as well as at four lower doses. The results of those experiments are illustrated below.

Figure 16:



Shown above are the dose-response curves for ^{14}C-AN covalent binding to rat globin (hemoglobin). Each point represents the mean ± S.E. for 3 - 6 rats. The points represent data collected from rats that were sampled when the covalent binding had reached completion at each dose. The lines connect the points plotted. Where no error bars are visible, they are smaller than the symbol.

Clearly Figure 16 shows that measurement of the incorporation of ^{14}C-AN into hemoglobin could be used as a biomarker of AN exposure in these rats but this requires the administration of ^{14}C-AN.

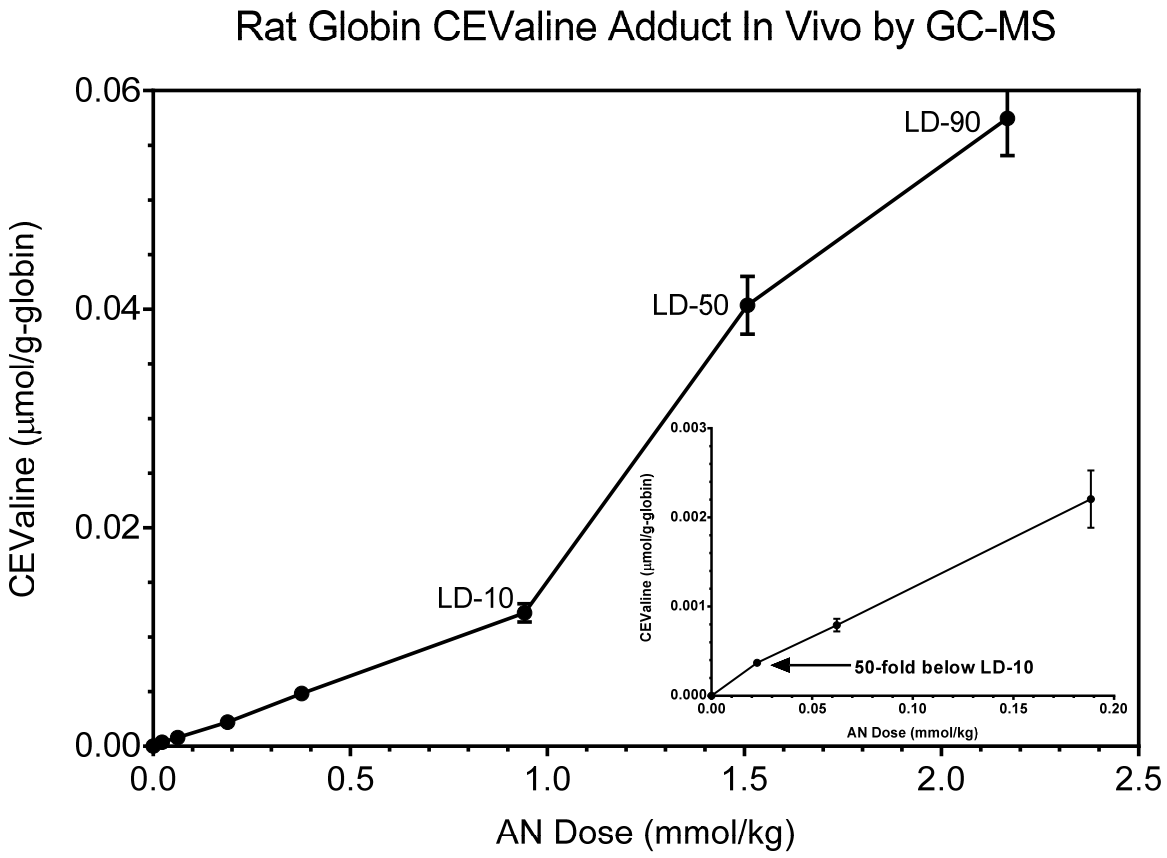
Another method for measuring the covalent incorporation of AN into hemoglobin as a biomarker for AN exposure that does not require the administration of ^{14}C-AN has been discussed in a previous Final Report for award W81XWH-08-1-0047 (page 11, W81XWH-08-1-0047 Final Report.pdf, submitted August 2010). An excerpt from the text of that report is repeated below for the convenience of the reader.

> *…….. hemoglobin, also being abundant in blood, has long been used to determine chemical adduct levels. The benefits of Hb adduct determination as an internal dose monitor for exposure were first suggested in 1976 by Ehrenberg and co-workers.*

Originally, adducts to histidine residues in Hb were determined in blood samples from chemically-exposed persons. However, application of this methodology was hampered by the complicated and tedious methods for sample preparation. A major improvement was realized in 1986 by Tornqvist and co-workers with a modification of the Edman-degradation method that made reliable determination of Hb adducts to the N-terminal valine by GC–MS feasible. Tornqvist, M., Mowrer, J., Jensen, S., and Ehrenberg, L. (1986).

We had previously applied this GC-MS method to the **same rat globin samples** as illustrated in Figure 16 (Benz et al (1997b). Those results are illustrated below in Figure 17.

Figure 17:



The **C**yano**E**thyl**V**aline-globin adduct levels increase with increasing AN dose as seen with ^{14}C-AN incorporation in Figure 16. Both methods can measure adducts at the lowest dose administered to these rats, namely 1.2mg/kg (0.023mmol/kg), which is approximately 50 fold lower than the LD10 of 50mg/kg (0.94mmol/kg).

The levels of the CEValine adducts (Figure 17) are some 500 fold lower than the levels determined by ^{14}C-AN incorporation (Figure 16). This large difference can be explained as described below.

Although it was suggested at that time that rat hemoglobin contained a highly reactive βCys-125, which would react with AN much faster than with the N-terminal valines and thus be monitored by ^{14}C-AN incorporation but not by the CEValine-GC-MS method, the actual differences in the rate constants were not known.

In our current project we have measured these rate constants for the reaction of AN with the N-terminal valines in human hemoglobin. As reported in Table 7, the average second-order rate constant for the α- and β-chain valines was 2.2E-02 $M^{-1}min^{-1}$. Although these measurements were made with human rather than rat hemoglobin, because these valines are on the surface of the protein in both species, any species difference in the rate constants is unlikely. This is supported by the fact that we have measured the second-order rate constants for the reaction of AN with Cysteine-93 in both human and rat hemoglobin and they are identical at 4.0E-2 $M^{-1}min^{-1}$ (Table 7).

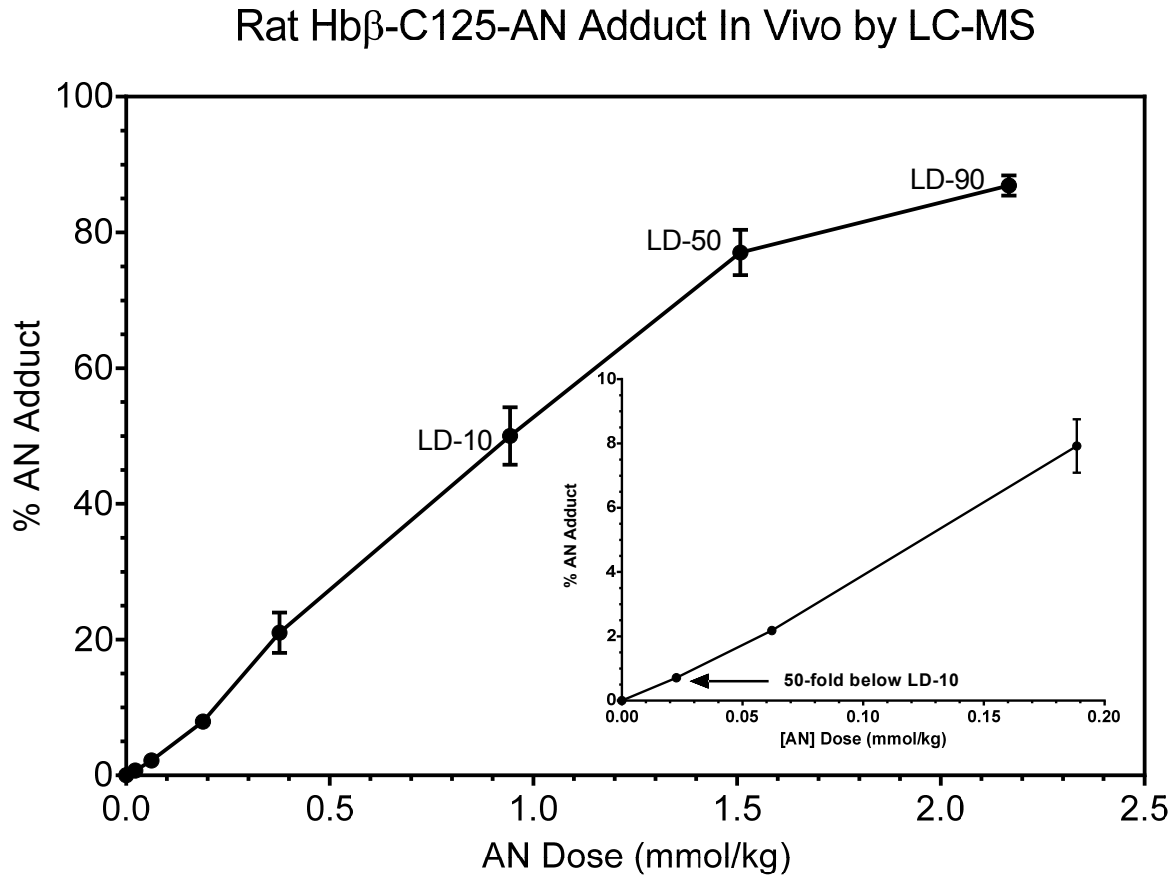
However, the rate constant measured for the reaction of AN with rat Hbβ-C125 was 23.9 $M^{-1}min^{-1}$, i.e. over 1000 times faster than the rate constant for the reaction with the N-terminal valines, thus the 500 fold difference in the adduct levels between Figure 16 and Figure 17 is understandable.

Although the GC-MS method for measuring CEValine-adducts of hemoglobin as a biomarker for AN exposure is a sensitive and accurate method, it is unfortunately time-consuming and thus not suitable for triage purposes. A major goal of this project was to develop an alternative biomarker that could be measured faster but with comparable sensitivity. The accomplishment of that goal was documented earlier.

To compare our new fast LC-MS method for measuring AN-adducts on reactive cysteines to the older and much slower GC-MS method for measuring AN-adducts on N-terminal valines as a biomarker for AN-exposure in rats we needed globin samples from rats exposed to AN.

Fortunately the globin samples that were originally used in the experiments illustrated in Figures 16-17 and reported in reference 1, were still available in our -70°C freezer. A summary of the LC-MS analysis of those samples is reported below. Detailed methodology for these analyses was presented on pages 6-12 of the Quarterly Report for the period October 1, 2012-December 31, 2012.

Figure 18:



Comparing the data in Figure 18 (LC-MS) with the data in Figure 16 (^{14}C-AN) indicates clearly that our LC-MS method yields a dose-response curve essentially identical in shape and with comparable sensitivity (both methods can detect adducts at the lowest dose administered) as the radioactive method.

Comparing the LC-MS method to the previous GC-MS method (Figure 17) again indicates comparable sensitivity but the GC-MS method for CEValine has a slope increase at doses above the LD-10. This slope change is related to the depletion of liver glutathione, which successfully competes for reaction with AN in vivo. At the LD-10 dose, all of the liver GSH is depleted and, as a result, the N-terminal valines in hemoglobin have significantly less competition for AN as the dose is further increased. See Benz et al 1997a, 1997b for details. This slope change in not reflected in the LC-MS or ^{14}C methods since the former only monitors the reaction of AN at Hbβ-C125 and although the latter monitors Total Covalent Binding to all sites in Hb, the reaction at Hbβ-C125 dominates the Total Covalent Binding monitored by ^{14}C . The reaction of AN with HbβC-125 is **faster** than the reaction of AN with GSH (Table 11) and thus is essentially independent of liver GSH.

It is important to recall that although the previous GC-MS method is specific and sensitive for measuring AN adducts in blood samples, it is also time consuming requiring more than one day to determine an adduct level. An important goal of this project was to develop a method that could assess human AN exposure from a blood sample rapidly enough to be used for triage purposes in a case of chemical warfare, a terrorist attack or and industrial accident. Table 9 provides a Time-Line indicating that we could process at least 10 blood samples within 2 hours and that further time optimization would be possible. However, all of our previous work was done on blood samples obtained from in vitro incubations of AN with human or rat blood. The results in Figure 18 indicate clearly that **in vivo** exposure to AN can be readily monitored with our methodology.

With regard to the sensitivity of the LC-MS method for monitoring in vivo samples, the lowest dose illustrated in Figure 15 was 1.2mg/kg (0.023mmol/kg), which is 50X lower than the LD-10 in rats. That was the lowest dose administered in those experiments over 15 years ago. Assuming that the AN-dose-response curve in rats (Figure 15) is predictive of the AN-dose-response curve in humans, the ability of the LC-MS method to measure adduct levels at exposures 50X lower than the LD-10 indicates that the method has sufficient sensitivity for triage purposes in humans.

However, we can justifiably predict that our LC-MS method could monitor rat (and human) exposures below 1.2mg/kg. In Figure 14 we showed that we could detect Hbβ-C125-AN subsequent to in vitro incubations of AN with rat blood at AN concentrations as low as 2.5μM. Some years ago we monitored the AN blood levels of rats treated with AN doses from 10 – 115mg/kg. In the lower dose range the peak AN blood level was approximately proportional to the dose administered. The peak blood level observed in rats treated with the 10mg/kg dose was 70μM. Linear extrapolation to lower doses predicts that a dose of 0.3mg/kg would produce a peak blood level of approximately 2.5μM. Thus we anticipate that our LC-MS method could detect adducts in AN-exposed humans at exposures over 150 fold lower than the LD-10 in rats.

These predictions are predicated on our observations that the reactivity of HSA-C34 in human blood and Hbβ-C125 in rat blood with AN in vitro is comparable. The data substantiating this statement was provided in Table 11 and Figure 14.

In addition, we have shown that the reactivity of RSA-C34 and Hbβ-C125 in rat blood with AN in vitro is comparable (Table 11 and Figures 12-13).

To verify that these relative reactivity measurements conducted in vitro can predict in vivo behavior, ideally would require having rat plasma samples to match the globin samples from the rats treated with AN as in Figures 16-18. Unfortunately, we did not have the foresight 15 years ago to save all matching plasma samples. However, two matching plasma samples were stored frozen for rats treated with the 50mg/kg and the 115mg/kg doses. Analysis of peptides T6 (RSA-C34-IAA) and T6* (RSA-C34-AN) from those samples was illustrated on page 14 of the Quarterly Report for the period October 1, 2012-December 31, 2012.

Based on the comparable second-order rate constants for the **in vitro** reaction of AN with rat Hbβ-C125 and RSA-C34 in rat blood (Table 11), we would predict comparable percentages of alkylation at these two sites in rats exposed to AN **in vivo**. The MS analysis of these two rat plasma samples bears this out. The 50mg/kg dose produced 50% alkylation on Hbβ-C125 and

43.6% alkylation of RSA-C34, whereas the 115mg/kg dose produced 86.9% alkylation of Hbβ-C125 and 80.6% alkylation on RSA-C34.

Quarter2-NCE:

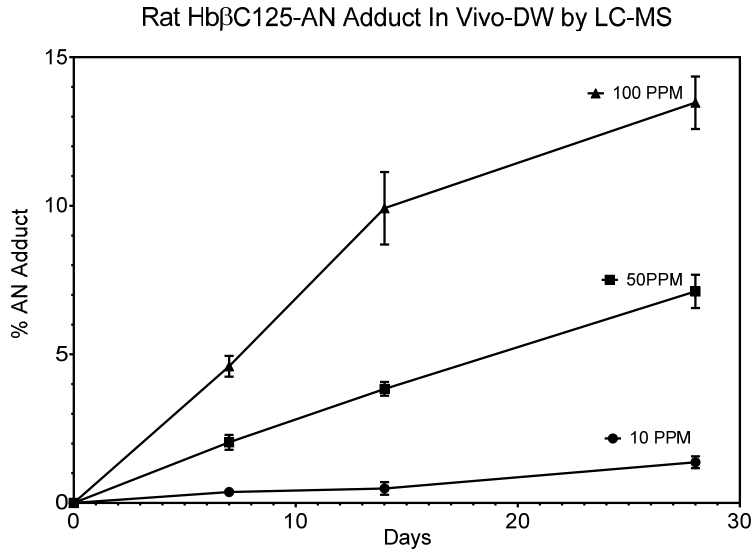
During this quarter we expanded the use of our LC-MS method beyond assessment of acute AN exposures to chronic AN exposures. Again fortuitously, we have frozen rat globin samples from a series of rat experiments conducted some years ago where the rats were exposed to AN in their drinking water from 0 to 28 days. In this quarter we assessed the levels of AN-adducts in these blood samples.

In addition, we had the fortunate opportunity to access 20 plasma samples from employees at a local industrial plant that uses acrylonitrile in their production of polymers, elastomers and specialty chemicals. We identified one individual of the 20 whose AN-adduct level was over 26 times higher than the average of the other 19 employees. This is exactly the type of high exposure that our assay was designed to discover.

Assessment of rHbβC125-AN adducts in rats chronically exposed to AN in drinking water. A detailed description of the methodology used for these measurements was presented on pages 2-6 in the Quarterly Report for the period January 1, 2013-March 31, 2013.

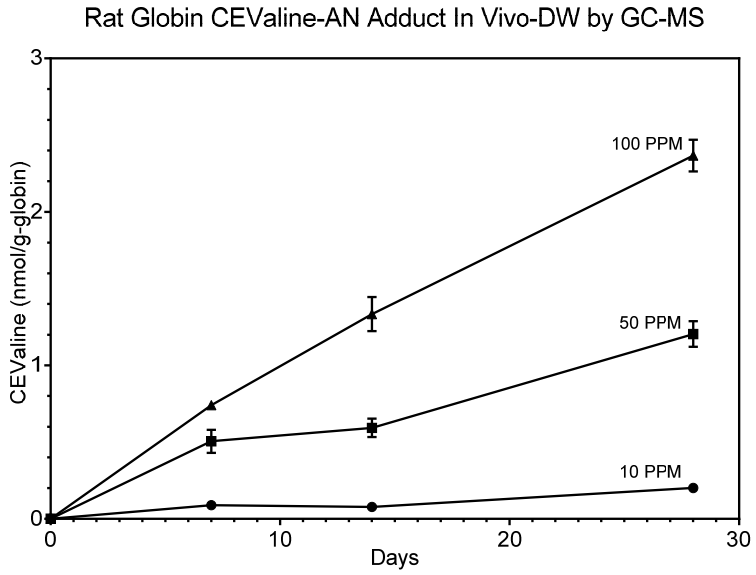
Figure 19 provides a summary of the results of those experiments. It illustrates a plot of the % HbβC125-AN adduct as a function of the concentration of AN in the drinking water and the time of exposure

Figure 19:



These identical samples had been analyzed over 10 years earlier by the laborious and time consuming GC-MS method for quantification of the N-terminal **C**yano**E**thyl**V**aline-AN adduct. The data obtained at that time by that method is illustrated below in Figure 20.

Figure 20:



Comparing the LC-MS plots in Figure 19 to the GC-MS plots in Figure 20 indicates comparable shapes as a function of AN-exposure concentration and time. However, if we convert the % adduct levels for rHbβC125-AN in Figure 19 to nmol/g-globin, as for the CEValine-adducts in Figure 20, we find that the C125-AN adducts are over 1000-fold higher. This difference is predictable from the ratio of the second-order rate constants for the reaction of AN with rat C125 vs. the N-terminal valines as presented in Table 7.

The results in Figure 19 indicate clearly that **chronic in vivo** exposure to AN can be readily monitored with our methodology and Figure 18 indicates the same is true for **acute in vivo** exposure.

AN-Exposure Levels monitored in AN Industrial Plant Employees:
As a result of this project, we have developed a rapid and sensitive assay for AN exposure in humans and we were eager to apply this methodology to real-world samples. We are fortunate to have at our university a group of faculty members who have an Occupational Health Surveillance Project that has been in place for decades. Employees at several industrial chemical plants here in Louisville are monitored routinely throughout their work life at the plants. Blood and plasma are collected and banked in -70°C freezers.

We requested and were granted status as key personnel in these projects by the University of Louisville IRB. The IRB approval letters were attached as an appendix to the Quarterly Report for the period January 1, 2013-March 31, 2013.

Employees are ranked by the Occupational Health Surveillance Project with respect to their likelihood for exposure to AN based on their job description. The ranking is from 1-6, where 1 represents low exposure likelihood and 6 represents high likelihood for exposure. We requested samples from employees at a plant where AN is one of the primary chemicals used, 10 employee

samples with a rank of 1 and 10 with a rank of 6. In addition, since AN is a constituent of tobacco smoke and smokers are known to have AN-protein adducts in their blood, we requested that half of the samples from each rank come from smokers and non-smokers.

These 20 samples were analyzed for HSA-C34-AN by our LC-MS method. The methodology used and detailed results were presented on pages 9-11 in the Quarterly Report for the period January 1, 2013-March 31, 2013.

Using the sensitive and highly selective MRM approach described in that Quarterly Report we analyzed all 20 employee samples. The data collected are shown the Table 12 below.

Table 12:

Sample	Albumin Cys34-AN Adduct Percent	Albumin Cys34-AN Adduct nmol/g albumin	Smoking	Acute Exposure	CERM
FWB	0.0	0.0	Non Smoker	0	
FWB	0.0	0.0	Non Smoker	0	
20975	0.0	0.7	Non Smoker	1	1600.8
27161	0.1	21.5	Non Smoker	1	1091.2
2675	0.2	25.2	Non Smoker	1	595.8
4836	0.3	41.4	Non Smoker	1	916.1
4244	3.0	457.3	Non Smoker	1	1347.6
20909	0.0	0.0	Smoker	1	852.3
25684	0.0	0.1	Smoker	1	1059.5
25365	0.0	0.3	Smoker	1	658.6
20868	0.0	1.0	Smoker	1	897.3
20896	0.0	5.1	Smoker	1	727.2
27072	0.0	1.9	Non Smoker	6	774.4
21109	0.1	21.4	Non Smoker	6	528.6
25420	0.2	24.7	Non Smoker	6	1004.3
5746	0.3	48.2	Non Smoker	6	788.8
25429	0.0	0.4	Smoker	6	693.3
25997	0.0	0.9	Smoker	6	456.8
26450	0.0	2.1	Smoker	6	441.9
26574	0.1	12.0	Smoker	6	493.1
25909	0.1	12.8	Smoker	6	525.5
25366	0.7	106.2	Smoker	6	1025.0

Most employees had low levels of AN-adduct. However, one employee (4244) had an AN-adduct level over 26 times higher than the average of the other 19 employees and over 4 times higher than the next highest level (25366). This is exactly the type of aberrant AN-exposure that our method was developed to detect!

With regard to the other samples analyzed, there is no obvious correlation of an employees' AN-adduct level with either their Acute Exposure Rank or CERM (Cumulative Exposure Rank Month) values, which are applied to the employees by the Occupational Health Surveillance Project based on their occupational job description within the plant.

With regard to the data in Table 12, several important points need to be made. First we have upmost confidence in the AN-adduct levels we have measured in these employees, even at the very lowest levels. The sample FWB in Table 11 represents blood drawn from the PI of this project and the author of this report, who has no exposure to AN and whose adduct levels were undetectable. This indicates that our LC-MS method is applicable to both high and low exposure levels.

Second, the MRM method used on these samples is the 'gold-standard" for quantification because of its specificity. Third the half-life of human albumin in blood is approximately 3 weeks and thus any adducts measured in these employee samples will only reflect their exposure to AN over a few months prior to their blood sampling. This is especially important with regard to the lack of correlation of the measured AN-adduct levels and the CERM data as the latter is an index of exposure over the total working life of the employee in the plant.

In conclusion, in this quarter we have demonstrated that our LC-MS method is effective for measuring AN-adducts on blood proteins in animals treated chronically with AN in their drinking water and that an AN-plant worker was detected with aberrantly high HSA-C34-AN adduct level in a blood sample and that the reason for that exposure should be investigated. It is exactly that type of aberrant exposure that our method was designed to detect.

Quarter3-NCE:

During the last quarter of this project we began experiments directed toward understanding the mechanism behind the acute toxicity of AN. If the mechanism of AN-toxicity can be deciphered, then countermeasures (antidotes) can be developed directed towards these mechanisms.

Previous work in our laboratory indicated that there are two main components to the acute toxicity of AN. First is that it can be metabolized in the body to cyanide, a well-known acute toxin. However, we have previously shown that blocking cyanide production with inhibitors of the enzyme(s) that convert AN to cyanide does not prevent acute toxicity but only delays the time to death. This indicates that there is another component to the acute toxicity other than cyanide. This component is undoubtedly related to the ability of AN to form covalent bonds with tissue proteins. Those tissue proteins that contain a reactive cysteine residue that is critical to its biological function are the likely candidates.

Our experiments this final quarter were directed toward identifying tissue protein targets. We had available bio-banked rat tissue samples that had been isolated from rats treated with both non-lethal and lethal doses of AN. To date we have only examined a liver tissue sample obtained from a rat that had been administered a LD90 of AN subcutaneously.

We employed the "Universal" sample preparation methodology of Mann and colleagues (2009) for these analyses.

Briefly, a 0.5g sample of frozen liver, isolated from a rat that had been administered an LD90 (115mg/kg) dose of AN, was homogenized in 5ml of 2% SDS, 0.1M Tris-HCl, pH 7.6 with a Ten Broeck homogenizer. A portion of the homogenate was removed for BCA protein analysis and dithiothreitol was added to the remaining portion to bring the DTT concentration to 0.1M, followed by a few more passes with the homogenizer. The lysate was then incubated in boiling water for 3 minutes.

A portion of the lysate was then placed in a Microcon 30K molecular filtration device and the SDS lysis buffer was exchanged to 50mM Tris/8M Urea, pH 8.5 via several buffer exchange/centrifugation steps.

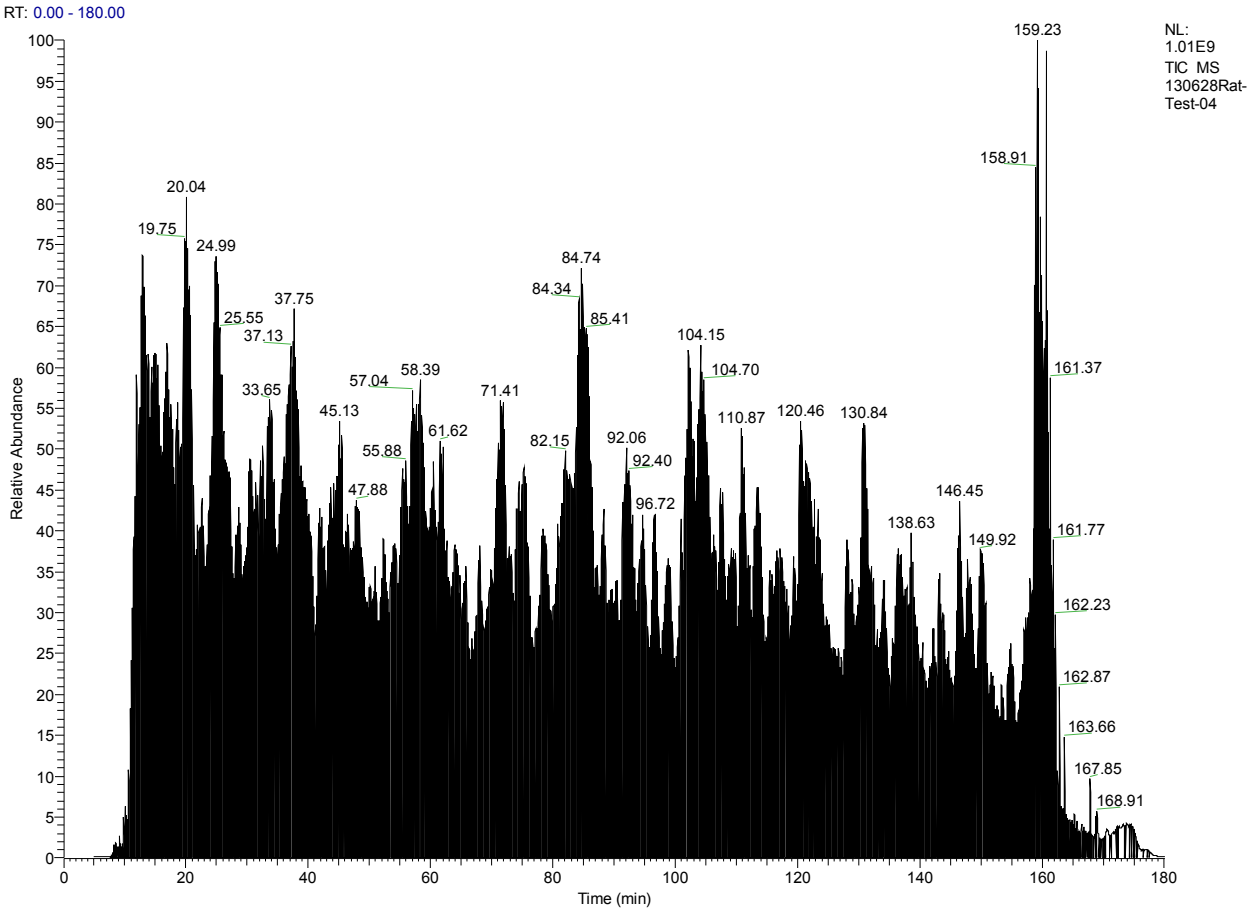
To the final buffer-exchanged protein concentrate (50ul), 50ul of 50mM iodoacetamide in 50mM Tris/8M Urea was added and the mixture was incubated in the dark for 15min to allow for alkylation of all the free cysteine residues in the protein extract.

Subsequently, the excess iodoacetamide and urea was exchanged for 50mM Tris/1M Urea, pH8.5 via several buffer exchange/centrifugation steps.

To the final protein concentrate (50ul), 200ul of Lys-C/Trypsin (4ug) from Promega was added and the mixture was incubated in a water bath overnight (19h) to digest the protein extract into peptides for further analysis.

The resulting complex peptide mixture was analyzed by 3 approaches. In the first approach, a portion of the digest was analyzed directly by nano Reverse Phase LC-MS with Data-Dependent Scan (nanoRPLC-DDS-MS). A typical Total Ion-Chromatogram resulting from this approach is illustrated below.

Figure 21:



This peptide digest, perhaps containing over 100,000 peptides, is much too complex for this approach in that analysis of this data yielded the identification of only 996 liver proteins, 50 of which contained covalently-bound AN.

There are two primary reasons for the low number of protein identifications from analysis of the data from this 1D approach. First is that there is a finite scan-time in the nanoRPLC-DDS-MS. If the peptides are poorly resolved, i.e. too many elute at the exact same retention time, there is insufficient time for the mass spectrometer to do an MS/MS scan on each of them. Applying preliminary fractionation using a first dimension separation technique decreases the number of peptides that co-chromatograph in the second dimension and thus increases the likelihood for sufficient MS/MS scan time, which following database search, can lead to identification of each peptide. The more efficient the separation of the peptides in the first dimension, the more likely peptides will be sufficiently resolved to trigger MS/MS scans in the second dimension. A second reason is that a greater peptide mass can be applied in the second dimension after preliminary fractionation as each of the fractions now contain only a portion of the original complex digest.

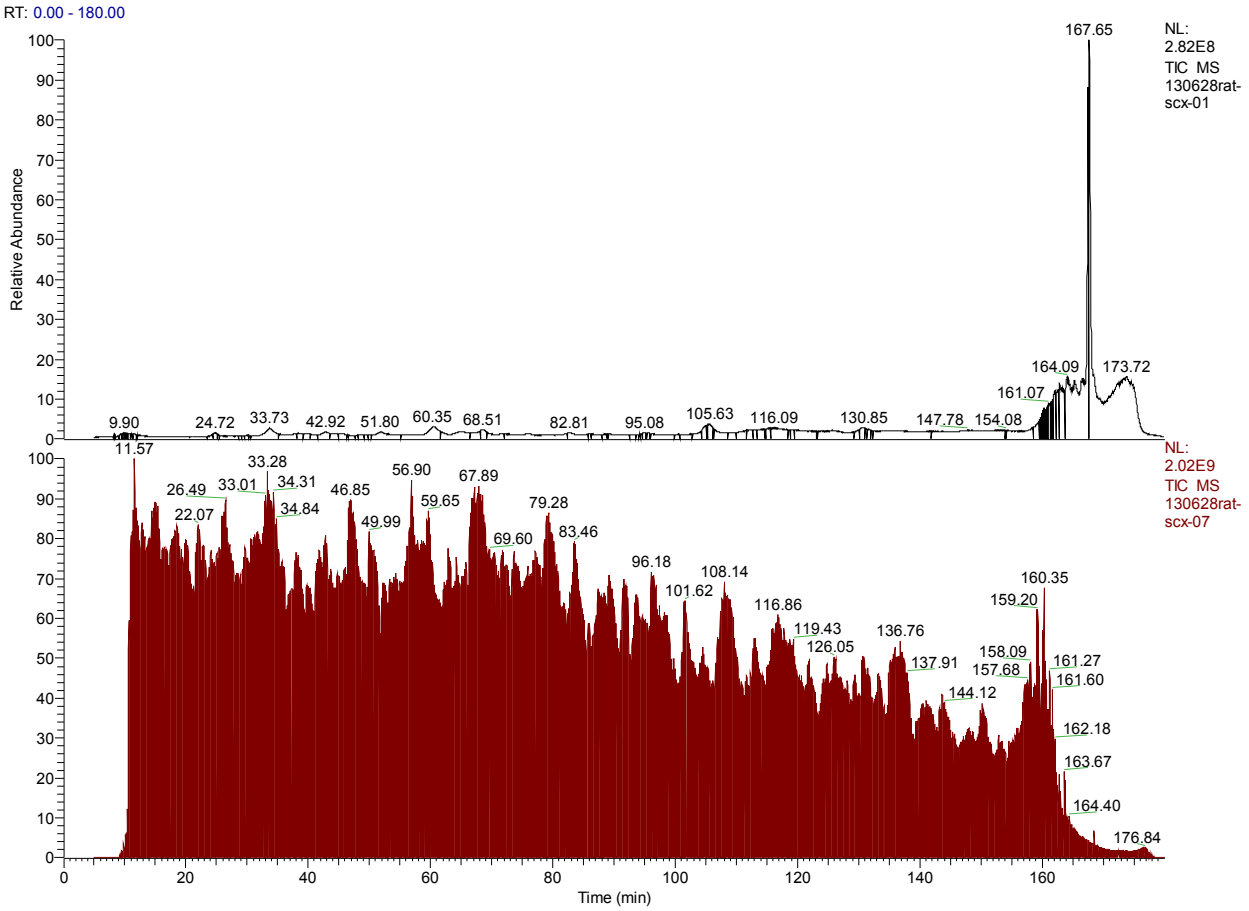
Based on this, these results were not unexpected as such complex digests normally are processed using two-dimensional techniques. One popular 2D technique utilizes an initial fractionation of

the complex peptide mixture by Strong Cation eXchange (SCX) chromatography followed by nanoRPLC-DDS-MS analysis of the individual SCX fractions. This was our second approach.

Briefly, a portion of the liver protein digest was applied to a Bruker Microtrap SCX column (1.0x8.0mm) and the peptides were eluted with step-wise increments of pH and ionic strength with buffers containing varying mixtures of 1.0M acetic acid, 1.0M ammonium acetate and water. A total of 16 fractions were collected and then each was then independently analyzed in the 2nd dimension by nanoRPLC-DDS-MS.

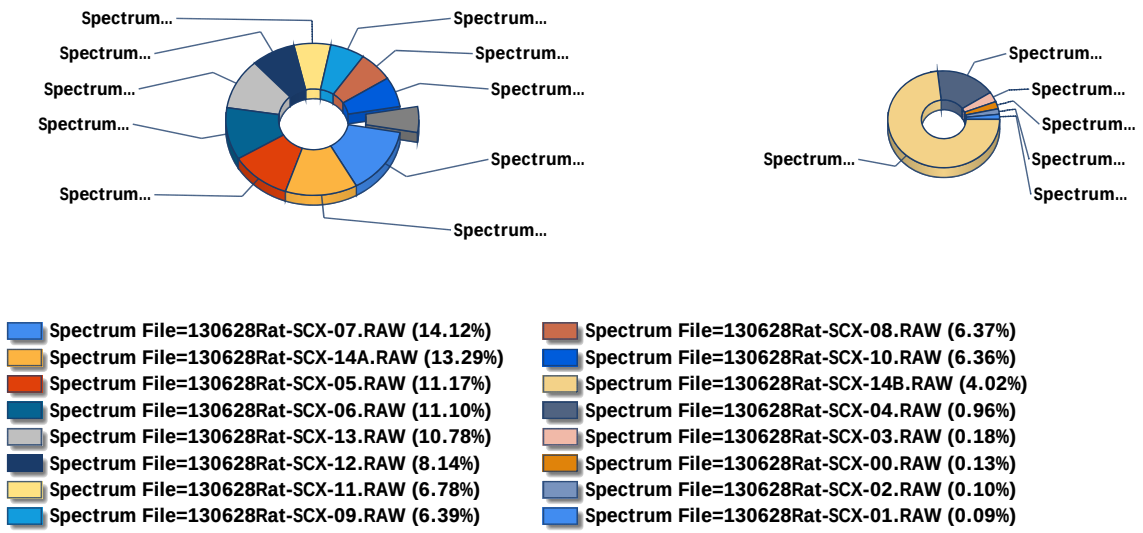
A 2nd dimension total ion chromatograms from two of these fractions is illustrated below.

Figure 22: (top Fraction #1, bottom Fraction #7)



It can be seen that this is very much a hit and miss approach in that some fractions may contain very few peptides (#1) while others contain an abundance of peptides (#7). A summary of the contribution of each of the 16 fractions (Spectrum File) to the total number of MS-MS spectra acquired is shown below.

Figure 23:



Notice one of the fractions (#7, SCX-07) contributed 14.12% to the total number of MS-MS spectra acquired whereas fraction #1, SCX-01 contributed only 0.09%. The greater the number of MS-MS spectra acquired in a given fraction, the greater the contribution of that fraction to the eventual identification of a protein following the search for those spectra in the MS-MS spectra database.

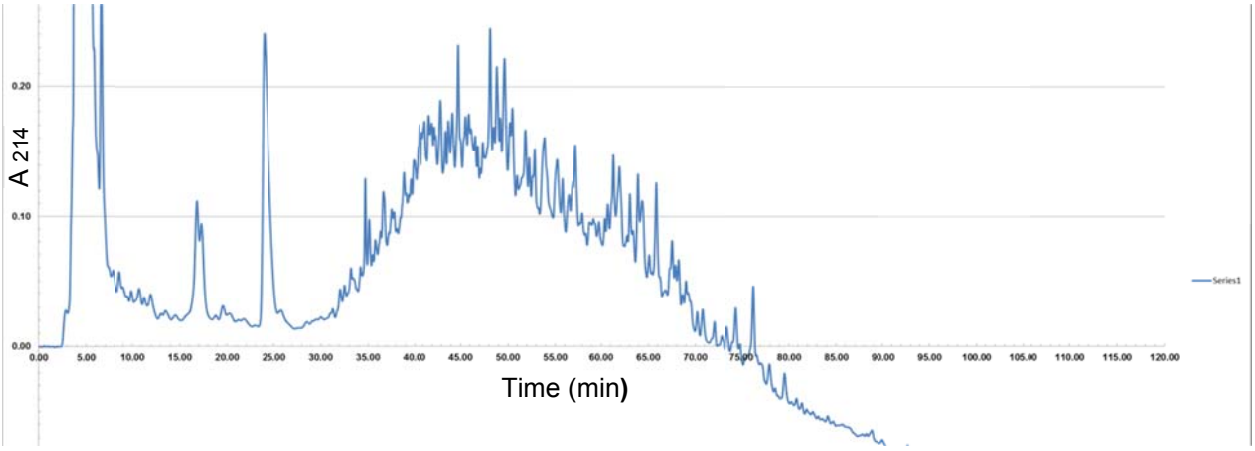
One does not know a priori what would be the best pH/ionic strength gradient to use on the SCX column to elute roughly equal numbers of peptides in each fraction, which would maximize the likelihood of their identification in the second dimension. One of course can optimize this by trial and error but this can be time consuming.

Despite the fact that in our initial SCX trial we obviously did not have optimum elution conditions for each fraction, this approach yielded the identification of 2,884 liver proteins of which 254contained AN-adducts.

Rather than immediately attempting to optimize the SCX approach, we instead decided to use an alternative 2D strategy that had been reported to yield even better results than the SCX method (Wang et al, 2011). This strategy involves using high pH RPLC in the first dimension, with multiple fraction concatenation. This strategy proved to be the most useful approach.

A typical 1st dimension high pH RPLC chromatogram (Waters XBridge 3.0x150mm) of the liver protein digest is shown below.

Figure 24:



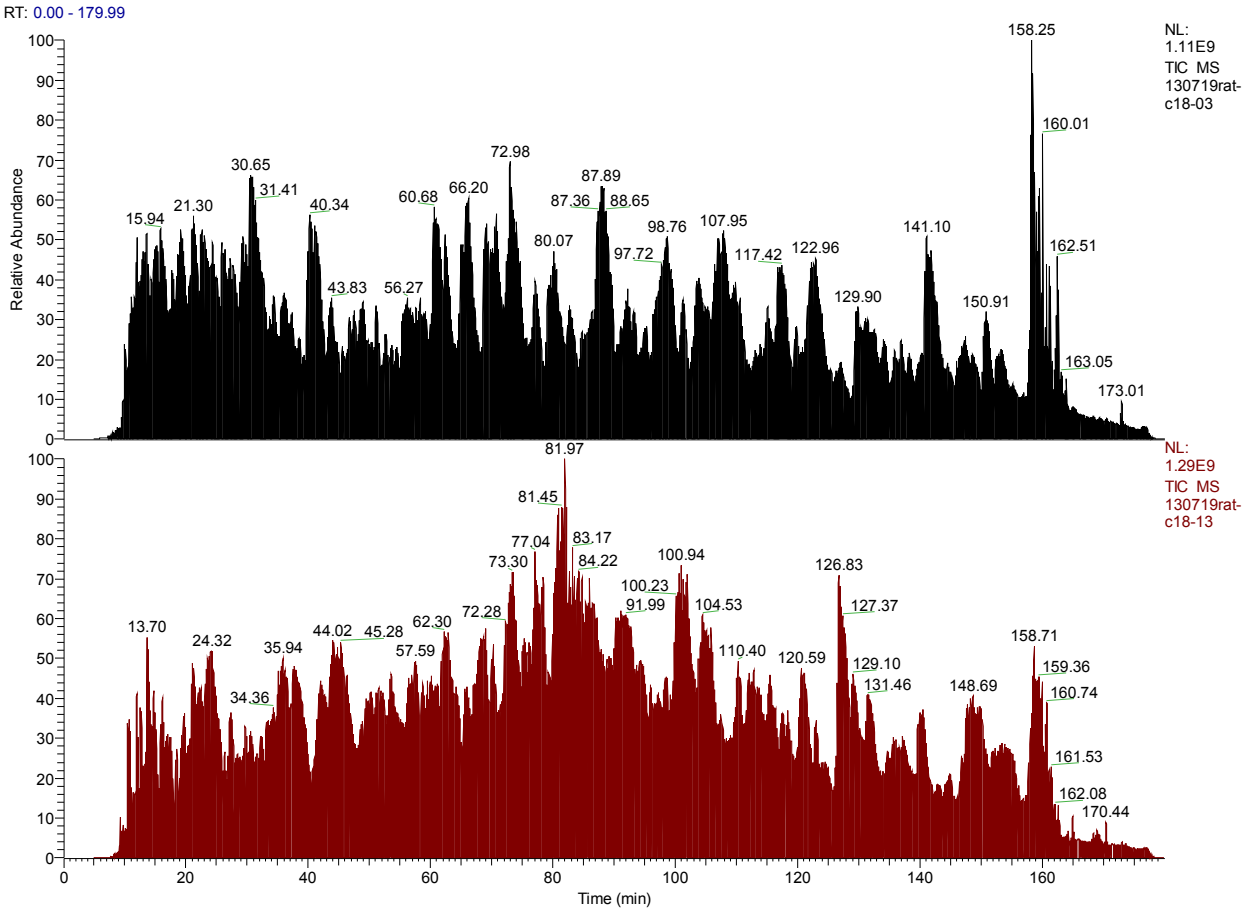
We used gradient elution to increase the separation of the peptides in this 1st dimension. Buffer A was 95% 10mM ammonium formate (pH 10.0)/5% ACN (acetonitrile), while Buffer B was 10% 10mM ammonium formate (pH 10.0)/90% ACN. The gradient was as follows: 98%A/2%B from 0-15min, 2%B-17%B from 15-30min (1%/min), 17%B-52%B from 30-100 min (0.5%/min) and 52%B-98%B from 100-123min (2%/min). The column flow rate was 0.2ml/min. Fractions were collected every 90 sec (0.3ml) beginning at 10 min through 100 min (60 fractions). The 60 fractions were concatenated into 15 fractions using the following table.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
46	47	48	49	50	51	52	53	54	55	56	57	58	59	60

The 15 individual fractions were dried in a speedvac, dissolved in 10ul of 5%ACN/0.1% Formic Acid and then individually analyzed by nanoRPLC-DDS-MS as done for the SCX fractions.

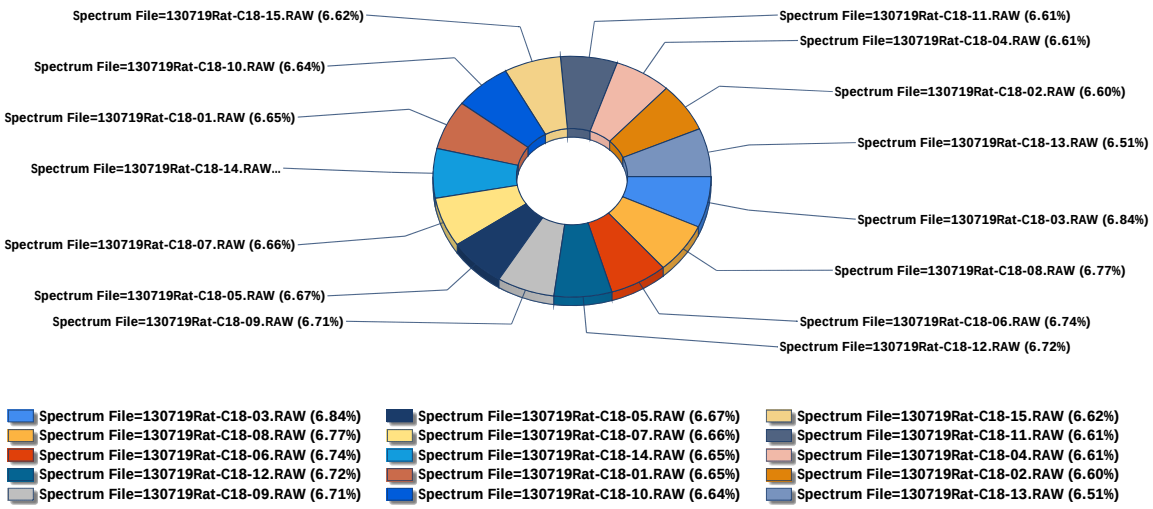
The superiority of the high pH RPLC 1st dimension approach was clearly demonstrated in that the peptides in the digest were uniformly distributed among the 15 fractions. A typical 2nd dimension ion chromatograms from concatenated fractions #3 and #13 are illustrated below.

Figure 25: (top Fraction 3, bottom Fraction #13)



As can be seen in the figure below, the difference between the fraction that was the highest contributor to the total number of MS-MS spectra acquired (C18-03, 6.84%) and the lowest contributor (C18-13, 6.51%) was only 0.33%! This difference was over 14% for SCX 1st dimension fractionation (Figure 23).

Figure 26:



This superior approach allowed us to identify 4227 rat liver proteins, 385 of which contained AN-adducts. The table below compares the results of the three different methods used.

Table 13:

Method	Proteins Identified	
	Total	AN-adducted
nanoRPLC-DDS-MS	996	50
SCX-nano-RPLC-DDS-MS	2884	254
High pH RPLC-nano-RPLC-DDS-MS	4227	385

These proteomic results are very recent and we have not had time yet to carefully review the biological functions of these 385 proteins and, more importantly, determine whether their adduction by AN would be anticipated to impair their function, which might lead to toxicity/lethality. However, we are very excited by these results and we look forward to "mining" this data to look for the toxic AN-needle(s) in this 385 protein haystack.

Key Research Accomplishments:

- identified the "universe" of reactive sites within human serum albumin (HSA) that react with acrylonitrile (AN)
- determined within this "universe" the five most reactive amino acid sites in HSA
- determined that the second-order rate constant for the reaction of AN with HSA Cysteine-34 (C34) is so fast that the other reactive sites within the molecule can be ignored
- determined the "universe" of reactive sites within human hemoglobin (Hb) that react with acrylonitrile
- determined within this "universe" the five most reactive amino acid sites on the α-chain and the six most reactive sites on the β-chain
- determined the second-order rate constants for the reaction of AN with these eleven amino acid sites and ranked them in the order of reactivity
- determined that the most reactive site in human Hb was βChain Cysteine-93 (C93), which was approximately twice as reactive as the amino-terminal valines that have been used previously as biomarkers of AN exposure
- determined that HSA C34 reacted with AN over three times faster than human Hb βChain C93 and thus should serve as the best biomarker for AN exposure
- determined that there is a linear relationship between HSA C34 AN-adduct levels and blood AN concentration up to approximately 0.25mM AN, beyond which the cysteine site approaches saturation.
- determined that HSA C34 AN-adduct levels could be detected with blood AN concentrations as low as 0.0025mM, which is approximately 1000 times lower that the AN blood level observed to be lethal in rats
- established that HSA-C34 AN blood adduct levels can serve as a sensitive biomarker of AN exposure in humans that can be used for triage purposes following a chemical attack or industrial or transportation accident involving acrylonitrile.
- developed a method for the direct injection of plasma for the measurement of human albumin-AN adducts thus bypassing the time consuming RPLC separation of tryptic peptides and greatly speeding up the analysis
- determined the optimal conditions for the rapid and complete digestion of human plasma for the measurement of human albumin-AN adducts thus accelerating the time consuming tryptic digestion and significantly speeding up the analysis
- determined that the "fast analysis" technique for measuring human albumin-AN adducts was as accurate and as reproducible as the RPLC method
- determined that the techniques developed for measuring human albumin-AN adducts could be readily applied to acrolein (AC) adducts
- determined that although the initial reaction of AC with HSA-C34 had the potential for subsequent additional reaction(s) due to it bifunctional nature, the adduct was actually quite stable in blood and thus could be used as a biomarker for AC exposure.
- determined that AC formed adducts with blood GSH as well as human Hbβ-C93 but that the HSA-C34-AN adducts would be a better biomarker for AC exposure

- determined the specific amino acid sites in rat albumin and rat hemoglobin with which AN would form adducts, namely RSA-C34 and rHbβ C125 and C93 as well as GSH
- determined the second-order rate constants for the reaction of AN with these sites in rat blood and compared those rates with those previously determined in human blood
- verified that these rate constants were indeed second-order rate constants as the first-order rate constants for the reactions were directly proportional to the AN concentration used
- determined the AN-concentration dependence of RSA-C34-AN and rHbβ-C125-AN and C93-AN adduct levels
- determined that the rHbβ C125-AN adduct was the appropriate biomarker for assessing rat exposure to AN, while HSA-C34-AN was the appropriate biomarker for assessing human exposure
- demonstrated that our LCMS methodology could be used to monitor AN-adduct levels in rats treated with a wide range of subcutaneous AN doses from 50-fold below the LD10 up to and including the LD90
- demonstrated that the above method was as sensitive as using 14C radioactive AN or the time consuming GCMS CEV-globin adduct method for monitoring these rat exposures
- established that we should be able to predict the expected human toxicity in AN-exposed individuals by measuring their HSA-C34-AN adduct levels in comparison to rat blood AN-adduct levels measure in AN-intoxicated rats, assuming roughly equal AN toxicity in both species
- demonstrated that our LCMS method could also be used to monitor AN-adduct levels in rats treated chronically with AN in their drinking water
- demonstrated that our LCMS method could be used to monitor AN-exposure in workers in plants using AN as a raw material in their production lines
- used our proteomic techniques to identify 385 rat liver proteins that were adducted by AN in rats administered an LD90 subcutaneously.

Reportable Outcomes:

Reportable Outcomes: Abstracts for these presentations are in the Appendix.

F. W. Benz, J. Cai, D. E. Nerland and H. E. Hurst. Biomarkers of acrylonitrile exposure: second-order rate constants for the reaction of acrylonitrile with the most reactive sites in human hemoglobin. Toxicological Sciences 126, No. 1-Supplement, 528 (2012).

Harrell E. Hurst, Jian Cai, Donald E. Nerland, and Frederick W. Benz. Numeric model estimation of kinetic parameters for parallel and sequential acrylonitrile-hemoglobin adduct formation using accurate mass proteomic data. Journal of the American Society for Mass Spectrometry Vol. 23 Supplement 1, 57 (2012).

F.W. Benz, J. Cai, D.E. Nerland, and H.E. Hurst. Evaluation of a Biomarker for Assessment of Acute Acrylonitrile Exposure in Humans. Toxicology Letters 211S: S47 (2012).

Manuscripts for journal publication of this work are in preparation.

Conclusions:

Our overall conclusion is that we accomplished virtually all of the goals listed in our original Statement of Work. These accomplishments are listed below.

Our Statement of Work for Year 1 on this project was as follows:

Year 1:

a) characterize the biomarkers in human blood of exposure to the TICs acrylonitrile and acrolein
b) determine which of these TIC-blood-protein/peptide adducts (biomarkers) is most suitable for exposure monitoring for each TIC
c) characterize the TIC blood concentration vs. adduct level response for these biomarkers in human blood
d) conduct studies aimed at minimizing the biomarker analysis time so that the methods will truly be suitable for triage purposes

We accomplished goals a) through c) for acrylonitrile.

Our Statement of Work for Year 2 on this project was as follows:

Year 2:

e) characterize the TIC blood concentration vs. adduct level response for this biomarker in rat blood

f) use the relationships determined in c) and e) to estimate the expected human toxicity for a given human biomarker level based on the known relationship between rat toxicity and rat blood biomarker level
g) investigate alternative mass spectral and possibly antibody-based technologies that would minimize the equipment needed for human blood biomarker measurement

We accomplished Year 1 goals a) through c) for acrolein. In addition we accomplished goal d) from Year 1 for acrylonitrile demonstrating that the mass spectrometric method we developed for measuring the HSA-C34-AN adduct in plasma is suitable for triage of exposed individuals resulting from an act of chemical terrorism or industrial or transportation accident involving acrylonitrile. Finally, we accomplished goal e) for acrylonitrile.

Nine Month No-Cost Extension:

During this extended period we accomplished Year 2 goal f) for acrylonitrile and began experiments directed toward identifying the mechanism of action of AN toxicity.

We accomplished these by making use of bio-banked samples of rat globin and plasma obtained from rats that had been either treated acutely with a range of doses of acrylonitrile subcutaneously or chronically in their drinking water. From these analyses we predicted that we could measure the biomarker for acrylonitrile exposure in humans from a level 150-fold lower than the LD10 in rats up to a lethal exposure. In addition, we applied our method to assess the acrylonitrile exposure levels in a group of employees in a local plant that uses acrylonitrile in its production line. This "real-world" application uncovered a single worker whose biomarker level indicated an AN-exposure 26 times higher than the average of the other workers. We used the final quarter of our award to begin work on perhaps the most interesting aspect of this project, namely the identification of the protein targets of acrylonitrile in tissues, one or more of which is/are at least partly responsible for the toxicity and perhaps carcinogenicity of acrylonitrile. Using proteomic approaches we identified 385 proteins in rat liver that has been adducted by AN in rats and we look forward to mining this proteomic data for clues to AN's mechanism of toxicity.

References:

Benz, F.W., D.E. Nerland, J. Li and D. Corbett. Dose-Dependence of Covalent Binding of Acrylonitrile to Tissue Protein and Globin in Rats. *Fundam. Appl. Toxicol.* 36:149-156 (1997a).

Benz, F. W., D.E. Nerland, D. Corbett and J. Li. Biological Markers of Acute Acrylonitrile Intoxication in Rats as a Function of Dose and Time. *Fundam. Appl. Toxicol.* 36:141-148 (1997b.

Tornqvist, M., Mowrer, J., Jensen, S., and Ehrenberg, L. (1986). Monitoring of environmental cancer initiators through hemoglobin adducts by a modified Edman degradation method. Anal. Biochem. 154 255-266.

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Appendices:

Abstract 1:

F. W. Benz, J. Cai, D. E. Nerland and H. E. Hurst. Biomarkers of acrylonitrile exposure: second-order rate constants for the reaction of acrylonitrile with the most reactive sites in human hemoglobin. Toxicological Sciences 126, No. 1-Supplement, 528 (2012).

Military and civilian personnel may encounter the challenge of dealing with an exposure to a toxic industrial chemical used in an act of chemical terrorism. Acrylonitrile (AN) is produced in large quantities by the chemical industry and is acutely toxic. Our objective is to define the chemical signatures of AN-adducts in human blood that will allow for the triage of exposed individuals. In this project we have measured the second order rate constants for the reaction of AN with the most reactive sites in human hemoglobin in vitro. Fresh human red blood cell lysates were incubated, under pseudo first-order conditions, with 100mM AN at 37C. As the reaction progressed, 10µl aliquots of the reaction mixture were quenched at various times in acidified acetone to precipitate the protein, the solvent was removed and the protein was alkylated with iodoacetamide and digested with trypsin. The tryptic digests were analyzed using an Accela LC System coupled to a LTQ-Orbitrap XL mass spectrometer. Both the appearance of AN-adducted tryptic peptides and the disappearance of the equivalent unreacted tryptic peptides were monitored. The eleven most reactive sites were monitored and the second-order rate constants for the initial AN-adduction were measured, although multiple adduction at the same site, e.g. at lysine, was detected. The slowest second-order rate constant measured was 1.0E-3 M-1min-1 for aK16. The increasing order of reactivity at the other sites relative to this slowest site is as follows: aK16(1.0) < bK17(1.2) < aH20(2.5) < aK11(4.6) < aK7(5.0) < bH97(5.1) < bK59(5.3) < bK8(6.0) < bV1(21.0) < aV1(23.0) < bC93(40.0). As can be seen, the most reactive site for AN-adduction in human hemoglobin was bC93. Its rate of adduction was approximately twice that of the N-terminal valines, which have often been used as a biomarker of AN exposure. Supported by DOD, U.S. Army Medical Research and Material Command, W81XWH-10-2-0143.

Abstract 2:

Harrell E. Hurst, Jian Cai, Donald E. Nerland, and Fredrick W. Benz. Numeric model estimation of kinetic parameters for parallel and sequential acrylonitrile-hemoglobin adduct formation using accurate mass proteomic data. Journal of the American Society for Mass Spectrometry Vol. 23 Supplement 1, 57 (2012).

As many industrial chemicals and metabolites react with proteins following exposure, measurements of chemical adducts to hemoglobin (Hb) have been used for assessment of systemic exposure to reactive chemicals. Mass spectrometry enables sensitive detection of specific sites of chemical adduct formation, which can be used for determination of extent of chemical exposure. Knowledge of adduct formation kinetics is important in MS measurements of peptides with multiple potential adducts, as transient products form and progress to other adduct products by sequential or parallel routes with continuous exposure. We present means for

optimized determination of pseudo first-order rate constants for formation of multiple specific acrylonitrile (AN) adducts in a reaction network involving the N-terminal tryptic peptide of β-globin during increasing exposure time.

Fresh human red blood cell lysates were incubated with 100mM AN at 37□C. 10µl aliquots of reaction mixture were withdrawn and precipitated in acidified acetone at 10 time points over 24h. Solvent was removed, protein was alkylated with iodoacetamide and digested with trypsin. Tryptic peptides were analyzed using an Accela LC - LTQ-Orbitrap XL mass spectrometer. Both appearance of AN- peptide adducts and disappearance of unreacted tryptic peptides were monitored. Kinetics of adduct formation in the tryptic peptides βT1 or βT1-2 including residues 1-8 in the Hb β-chain were quantified by label free methods. Pseudo first-order rate constants were estimated by simultaneous integration of rate equations and optimization of the kinetics using acslX software in a reaction network model.

Summation of total areas of multiply charged peaks (z=1,2,3) evident in tryptic peptides βT1 or βT1-2 provided quantifiable signals. Peptide βT4 contained no amino acids reacting with AN, and summation of areas (z=2,3) was used for standardization of relative response of peptide adducts . Observed changes in βT1 and βT1-2 peptide adduct levels indicated the following reactions (rate constants), which were included in the reaction network model: Hb -->AN-V1(k1), Hb -->AN-K8(k2), AN-V1 -->AN-V1-AN-K8(k3), AN-K8 -->AN-V1-AN-K8(k4), AN-V1-AN-K8 -->AN-V1-2AN-K8 (k5), AN-K8 -->2AN-K8(k6), and 2AN-K8 -->AN-V1-2AN-K8 (k7). Here V1 indicates the N-terminal valine and K8 is the eighth amino acid lysine, while AN- and 2AN- indicate single and double acrylonitrile adducts, respectively. As reactant disappearance and product appearance were both monitored, comparisons of rates allowed checks of reaction stoichiometry. Areas converted to percentages of initial reactant indicated the preponderance of the βT1-AN-V1 adduct at about 50% after 24h, while other final adduct levels were AN-V1-AN-K8 (19%), AN-V1-2AN-K8 (17%), 2AN-K8 (3%), and AN-K8 (2%). Use of asclX software for parameter determination using maximum log likelihood estimation with simultaneous numerical integration of modeled pseudo first order rate constants gave the following values k1=1.9e-3, k2=7.6e-4, k3=2.7e-4, k4=3.6e-3, k5=1.3e-3, k6=4.5e-6, and k7=4.2e-4 /min. Graphical presentation of calculated adduct concentrations at various times as points with model predictions as solid lines enabled visualization of the fit of the reaction network model. Variability of parameter estimates, as indicated by standard deviations of rate constant estimates, ranged from lows of 1 – 2 % for rate constants k1 and k2 to greater than 100% for rate constants k6 and k7. Supported by DOD, U.S. Army Medical Research and Material Command, W81XWH-10-2-0143.

Abstract 3:

F.W. Benz, J. Cai, D.E. Nerland, and H.E. Hurst. Evaluation of a Biomarker for Assessment of Acute Acrylonitrile Exposure in Humans. Toxicology Letters 211S: S47 (2012).

Acrylonitrile (AN) is produced in large quantities by the chemical industry and is acutely toxic. Preparedness for acts of chemical terrorism and warfare requires that methods be developed to assess the level of chemical exposure to allow for proper diagnosis and treatment of significantly exposed individuals. Our objective is to define the chemical signatures of AN-adducts in human

blood and to develop a rapid screening technology to enable triage of individuals following a known or suspected exposure.
Fresh human blood was incubated with AN in vitro at 37C, pH 7.4. Eleven individual amino acid sites of AN adduction in hemoglobin (Hb), nine sites in albumin (HSA) and the GSH-AN adduct were monitored by LC/MS using a LTQ Orbitrap XL mass spectrometer with an ESI source.
Second-order rate constants for the reaction of AN with most of these sites were measured. The most reactive of these 21 sites was found to be HSA-C34 at 13.5 M-1min-1, which was 3 times faster than the reaction with GSH and over 300 times faster than Hbβ-C93, the next two most reactive sites. There was a linear relationship between the level of the HSA-C34-AN adduct and the concentration of AN in the incubation from 0.0025 to 0.25mM, above which non-linearity was observed. Experiments directed toward optimization of sample preparation and MS-analysis time resulted in requiring less than 120 min for 10 analyses. We conclude that this assay for HSA-C34-AN adducts should serve as a suitable method for assessment of acute AN exposure in humans. Supported by DOD, U.S. Army Medical Research and Material Command, W81XWH-10-2-0143.

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